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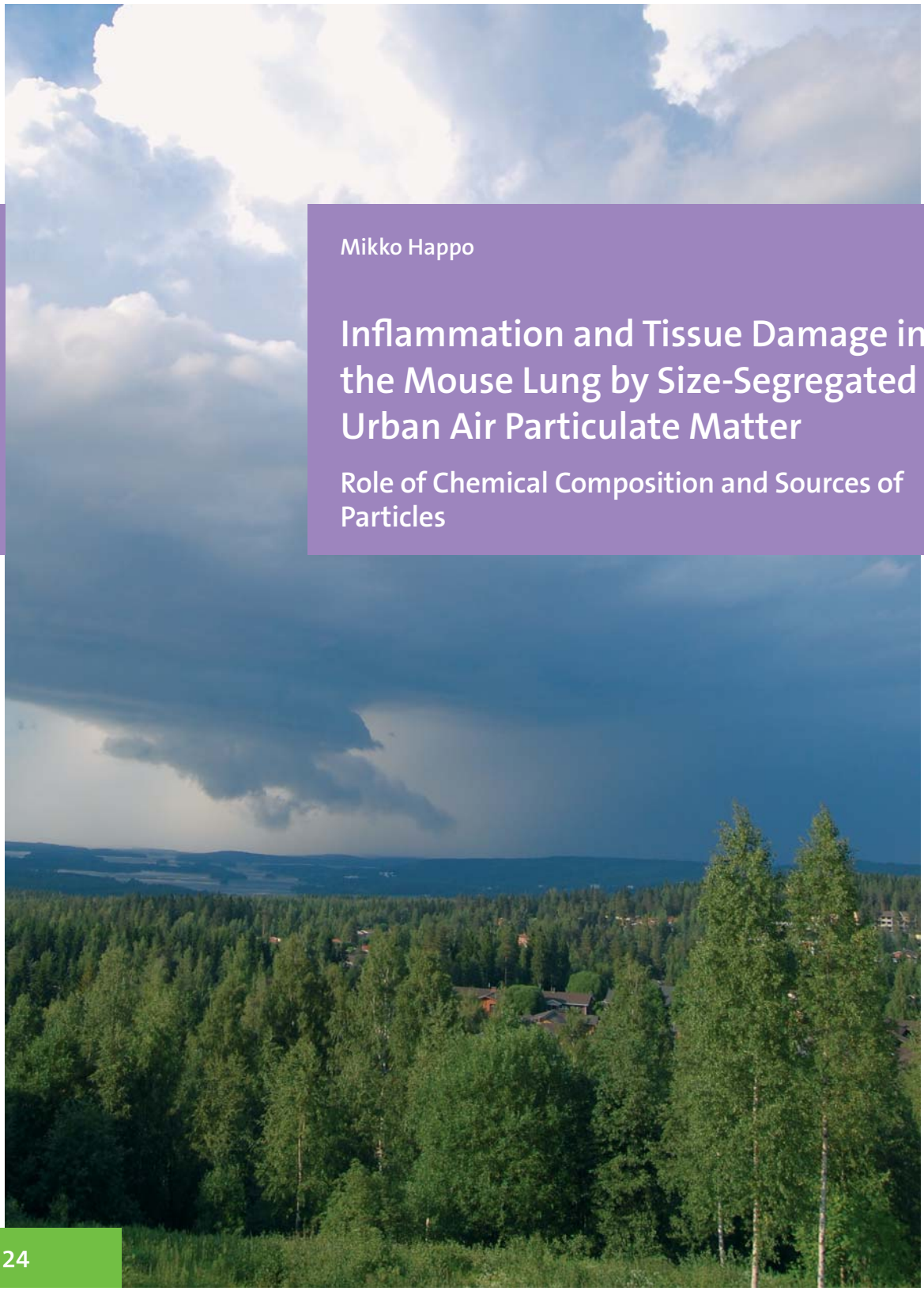

RESEARCH

Mikko Happo

Inflammation and Tissue Damage in the Mouse Lung by Size-Segregated Urban Air Particulate Matter

Role of Chemical Composition and Sources of Particles

Mikko Happo

INFLAMMATION AND TISSUE DAMAGE IN THE MOUSE LUNG BY SIZE-SEGREGATED URBAN AIR PARTICULATE MATTER

ROLE OF CHEMICAL COMPOSITION AND SOURCES OF PARTICLES

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Natural and Environmental Sciences, University of Kuopio, for public examination in auditorium, Tietoteknia Building, on December 4th, 2009 at 12 o'clock noon.

Department of Environmental Health,
National Institute for Health and Welfare, Kuopio, Finland
and
Faculty of Natural and Environmental Sciences, University of Kuopio, Finland

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To my family

ABSTRACT

Mikko Happo. Inflammation and tissue damage in the mouse lung by size-segregated urban air particulate matter – role of chemical composition and sources of particles. National Institute for Health and Welfare (THL), Research 24. 192 pages. Helsinki 2009
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Particulate air pollution is a global problem which has been shown to be responsible for a large variety of health outcomes. Current levels of urban air thoracic particles (PM_{10}; diameter <10μm) and more strongly fine particles ($PM_{2.5}$; diameter <2.5μm) have been associated with increased mortality and morbidity in urban populations. In addition to size and mass concentration, the chemical composition of particulate matter is important in evoking the adverse health effects. Immunotoxicity, such as inflammation, has been proposed as the main mechanism behind exacerbation of cardiorespiratory diseases. The aim of this dissertation was to investigate the immunotoxic properties of size-segregated particulate samples in relation to different source environments and chemical compositions.

Coarse ($PM_{10-2.5}$), intermediate ($PM_{2.5-1}$), accumulation ($PM_{1-0.2}$) and ultrafine ($PM_{0.2}$) particulate samples were collected during selected seasons and different air pollution situations in six European cities using a high volume cascade impactor (HVCI). The size-segregated particulate mass was collected on polyurethane foam substrates and a glass-fibre backup filter. The sampled mass was extracted with methanol and the samples in each campaign were pooled together according to the size range and the respective sampling site. In most studies, a fine particulate ($PM_{2.5-0.2}$) sample was formed by pooling the $PM_{2.5-1}$ and $PM_{1-0.2}$ samples together. Extensive chemical analyses of inorganic and organic constituents were made, which enabled chemical mass closure of the pooled $PM_{10-2.5}$ and $PM_{2.5-0.2}$ samples for each campaign.

The inflammatory potency of particulate samples was investigated by exposing the lungs of healthy C57BL/6J mice via intratracheal instillation. After 4 - 24 hours from the exposure, total cell number, cell differential, total protein and lactate dehydrogenase concentrations as well as the concentrations of proinflammatory cytokines (TNF-α, IL-6) and chemokine (KC) were detected from the bronchoalveolar lavage fluid (BALF) of the mice. The structural changes in the lung tissue were histopathologically assessed.

In all the studies, $PM_{10-2.5}$ samples had the highest inflammatory potency as assessed on the basis of the biochemical and cytological markers measured from BALF. However, there was more heterogeneity between sampling campaigns in the responses induced by the $PM_{2.5-0.2}$ samples. Particulate samples in $PM_{1-0.2}$ and $PM_{0.2}$ size-ranges showed negligible inflammatory activity. The present studies detected the appearance of

substantial amounts of the biochemical markers of inflammation in BALF already at 4 hours after the exposure, whereas the inflammatory cells appeared at 12 hours after the exposure. Thus, an awareness of the time-course of responses in the biochemical and cytological parameters measured in BALF is crucial if one wishes to achieve a reliable comparison of the inflammatory activities of urban air particulate samples collected in different locations or seasons.

$PM_{2.5-0.2}$ samples collected in warm and dry season field campaigns in Europe (Helsinki, Barcelona, Athens) were more potent inducers of inflammatory responses than those collected in cool and wet season campaigns (Duisburg, Prague, Amsterdam). This was apparently due to several reasons related to the major sources of air pollution and the climatic conditions during the sampling campaigns. In addition, spring, summer and autumn $PM_{2.5-1}$ samples collected at the same sampling site in Helsinki possessed clearly higher inflammatory activities in the mouse lung than the corresponding winter sample.

The increased inflammatory activity induced by fine particles was associated with oxidized organic compounds and transition metals, especially those derived from fuel oil combustion (Ni, V). Moreover, non-combustion emissions from vehicles and resuspended mineral dust displayed similar contributions to the inflammatory activity of both the fine and coarse particulate samples. Secondary inorganic ions (NH_4^+, NO_3^-, SO_4^{2-}) from regional and long-range transport had either negative or inconsistent associations with the inflammatory activity of the six-city $PM_{2.5-0.2}$ or the Helsinki $PM_{2.5-1}$ and $PM_{1-0.2}$ samples. Poor biomass and coal combustion were associated with elevated PAH contents in the fine particulate samples as well as an immunosuppressive effect in the mouse lungs.

The present studies revealed that particle size, sources and atmospheric transformation processes, such as photochemical oxidation, affect the inflammatory activity of and the duration of the response to urban air particulate matter in the mouse lungs. Local sources of oil combustion, and traffic-derived brake and tyre dust and resuspended mineral dust are likely to contribute to the inflammatory activity of urban air fine and coarse particles in conjunction with meteorological factors such as the intensity of sunlight, temperature and rain. Products from incomplete wood and coal combustion, especially PAHs, evoke different immunotoxic effects.

Keywords: Air pollution, particulate matter, mouse, inflammation, cytotoxicity, histopathology, particulate sources, particle size, chemical composition, chemical mass closure

TIIVISTELMÄ

Mikko Happo. Inflammation and tissue damage in the mouse lung by size-segregated urban air particulate matter – role of chemical composition and sources of particles. [Kokoluokiteltujen kaupunki-ilman hiukkasten aiheuttama tulehdus ja kudosvaurio hiiren keuhkoissa – Hiukkasten kemiallisen koostumuksen ja päästölähteiden merkitys]. National Institute for Health and Welfare (THL), Tutkimus 24. 192 pages. Helsinki 2009
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Hiukkasmaiset ilmansaasteet aiheuttavat monenlaisia terveyshaittoja maailmanlaajuisesti. Hengitettävien hiukkasten (PM_{10}; halkaisija <10 μm) ja niitäkin voimakkaammin pienhiukkasten ($PM_{2.5}$; halkaisija <2.5 μm) on todettu nykypitoisuuksissa lisäävän kuolleisuutta ja sairauskohtauksia kaupunkiväestön keskuudessa. Hiukkasten koon ja massapitoisuuden lisäksi hiukkasten kemiallinen koostumus vaikuttaa terveyshaittojen syntyyn. Immunotoksisuutta, erityisesti tulehdusta, pidetään pääasiallisena mekanismina hengitys- ja sydänsairauksien pahenemisessa. Väitöstutkimuksen tavoitteena oli tutkia erikokoisten hiukkasten immunotoksisuutta ja sen yhteyttä päästölähteisiin ja hiukkasten kemialliseen koostumukseen.

Karkeat ($PM_{10-2.5}$), välikokoiset ($PM_{2.5-1}$), kertymä- ($PM_{1-0.2}$) ja ultrapienet hiukkaset ($PM_{0.2}$) kerättiin suurtehokeräimellä (HVCI) eri vuodenaikoina ja erilaisista ilmanlaatutilanteista kuudessa eurooppalaisessa kaupungissa. Kokoluokiteltu hiukkasmassa kerättiin polyuretaanivaahtoliuskoille ja lasikuidusta tehdylle pohjasuodattimelle. Kerätty hiukkasmassa uutettiin metanolilla ja jokaisen kaupungin näytteet yhdistettiin kokoluokan ja keruupaikan mukaisesti. Useimmissa tutkimuksissa muodostettiin pienhiukkaskokoluokka ($PM_{2.5-0.2}$) yhdistämällä $PM_{2.5-1}$ ja $PM_{1-0.2}$ kokoluokkien näytteet. Yhdistetyistä HVCI-näytteistä tehtiin laaja orgaanisten ja epäorgaanisten yhdisteiden kemiallinen analyysi, joka mahdollisti kemiallisen massasulkeuman arvioimisen $PM_{10-2.5}$ ja $PM_{2.5-0.2}$ näytteistä.

Hiukkasnäytteiden kykyä aiheuttaa tulehdusta tutkittiin altistamalla terveiden C57BL/6J hiirten keuhkoja lyhytaikaisen nukutuksen aikana. Hiukkasnäyte annosteltiin näkökontrollissa hiiren henkitorven kautta keuhkoihin. Keuhkojen tulehdusreaktioita ja solujen vaurioitumista arvioitiin keuhkohuuhtelunäytteistä (BALF) tehtyjen sytologisten ja biokemiallisten määrityksien perusteella. BALF-näytteet kerättiin 4 - 24 tunnin kuluttua hiukkasaltistuksesta ja niistä mitattiin kokonaissolumäärä, soluerottelu, kokonaisproteiinin ja laktaattidehydrogenaasin pitoisuudet, sekä sytokiinien (TNF-α ja IL-6) ja kemokiinin (KC) pitoisuudet. Keuhkokudoksen rakenteelliset muutokset määritettiin histopatologisesti.

Jokaisessa osatyössä $PM_{10-2.5}$ näytteet tuottivat kaikkein voimakkaimmat tulehdusvasteet hiiren keuhkoissa, kun taas $PM_{2.5-0.2}$ näytteiden tuottamien vasteiden voimakkuudessa oli huomattavan suurta vaihtelua keräyspaikkojen välillä. Kaikkein pienimpien kokoluokkien ($PM_{1-0.2}$ ja $PM_{0.2}$) hiukkasnäytteet eivät tuottaneet merkittäviä tulehdusvasteita. Tutkimus osoitti selvästi, että suuria määriä tulehduksen biokemiallisia merkkiaineita ilmaantui keuhkoihin jo neljän tunnin kuluttua hiukkasaltistuksesta, kun taas tulehdussolut ilmaantuivat sinne vasta 12 tuntia altistuksen jälkeen. Tämän vuoksi määritysajankohdalla on ratkaiseva merkitys, kun pyritään luotettavasti vertailemaan eri paikoilta tai eri aikoina samalta paikalta kerättyjen kaupunki-ilman pienhiukkasten aiheuttamaa tulehdusaktiivisuutta toisiinsa.

Lämpimien ja kuivien mittauskampanjoiden aikana kerätyt $PM_{2.5-0.2}$ näytteet (Helsinki, Barcelona, Ateena) aiheuttivat voimakkaampia tulehdusvasteita kuin näytteet, jotka oli kerätty viileiden ja kosteiden mittausjaksojen aikana (Duisburg, Praha, Amsterdam). Tämä johtui useista tekijöistä, kuten erilaisista ilmansaasteiden lähteistä ja ilmastollisista olosuhteista hiukkasten keräyskampanjoiden aikana. Myös Helsingissä keväällä, kesällä ja syksyllä kerättyjen $PM_{2.5-1}$ näytteiden tulehdusaktiivisuus hiiren keuhkoissa oli selvästi voimakkaampaa kuin vastaavalla talvinäytteellä.

Pienhiukkasten kohonnut tulehdusaktiivisuus yhdistyi hapettuneisiin orgaanisiin yhdisteisiin ja erityisesti raskaan polttoöljyn poltosta peräisin oleviin siirtymämetalleihin (Ni, V). Liikenteen nostattama katupöly ja muut kuin palamisperäiset päästöt olivat yhteydessä samankaltaisiin kohonneisiin tulehdusvasteisiin niin pienhiukkasille kuin karkeille hiukkasille. Alueellisesta tai kaukokulkeumasta peräisin olevilla $PM_{2.5-0.2}$ ja $PM_{1-0.2}$ hiukkasten sekundäärisillä epäorgaanisilla ioneilla (NH_4^+, NO_3^-, SO_4^{2-}) oli joko negatiivisia tai epäjohdonmukaisia korrelaatioita näytteiden tuottamiin tulehdusvasteisiin. Huonoon biomassan ja hiilen polttoon liittyivät pienhiukkasnäytteiden kohonnut PAH-pitoisuus ja immunosupressiivinen vaikutus hiiren keuhkoihin.

Tämä väitöstutkimus osoittaa, että kaupunki-ilman hiukkasten päästölähteet, fysikaalinen koko ja ilmakehässä tapahtuva koostumuksen fotokemiallinen muutunta vaikuttavat hiukkasten aiheuttaman tulehduksen voimakkuuteen ja kestoon hiiren keuhkoissa. Paikallinen öljynpoltto, liikenteen jarru- ja rengaspöly ja mineraalipitoinen katupöly vaikuttavat kaupunki-ilman pienten ja karkeiden hiukkasten tulehdusaktiivisuuteen yhdessä säätekijöiden, kuten auringonvalon, lämpötilan ja sateen kanssa. Puun ja hiilen epätäydellisen palamisen tuotteet, erityisesti PAH-yhdisteet, aiheuttavat tästä poikkeavia immunotoksisia vaikutuksia.

Avainsanat: Ilmansaasteet, hiukkaset, hiiri, tulehdus, solutoksisuus, histopatologia, hiukkasten lähteet, hiukkaskoko, kemiallinen koostumus, kemiallinen massasulkeuma

CONTENTS

ABBREVIATIONS

BALF	Bronchoalveolar Lavage Fluid
CAPs	Concentrated Ambient Particles
COPD	Chronic Obstructive Pulmonary Disease
CXC	Chemokine containing two N-terminal cysteines separated by one amino acid
DA	Dicarboxylic Acids
DEP	Diesel Engine Exhaust
EC	Elemental Carbon
ELISA	Enzyme Linked Immunosorbent Assay
ED-XRF	Energy Dispersive X-Ray Fluoresence
GCMS-SIM	Gas Chromatograph Mass Spectrometer with Selected Ion Monitoring
HC	Hydrocarbon
HPLC-MS	High-Performance Liquid Chromatograph Mass Spectrometer
HVCI	High Volume Cascade Impactor
IC	Ion Chromatograph
ICAM	Inter-Cellular Adhesion Molecule
ICP/MS	Inductively-Coupled Plasma Mass Spectrometer
Ig	Immunoglobulin
IL-6	Interleukin 6
KC	Keratinocyte-Derived Chemokine
LAL	Limulus Amebocyte Lysate
LDH	Lactate Dehydrogenase
MCP	Monocyte Chemotactic Protein
MIP	Macrophage Inflammatory Protein
Nss-SO_4^{2-}	Non Sea Salt-Sulphate

OC	Organic Carbon
OE	Other Elements
PAF	Platelet-Activating Factor
PAH	Polycyclic Aromatic Hydrocarbon
PM_x	Particulate Matter with aerodynamic diameter less than x μm
PM_{x-y}	Particulate Matter with aerodynamic diameter between x and y μm
POM	Particulate Organic Matter
PUF	Polyurethane Foam
ROFA	Residual Oil Fly Ash
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
ΣMA	Monosaccharide Anhydrides
SS	Sea Salt
TGF-ß	Transforming Growth Factor Beta
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor Alpha
TOA	Thermal Optical Carbon Analyser
UM	Unidentified Matter
VEGF	Vascular Endothelial Growth Factor
WIS	Water-Insoluble Soil
WSS	Water-Soluble Soil

LIST OF ORIGINAL PUBLICATIONS

I Happo MS, Salonen RO, Hälinen AI, Jalava PI, Pennanen AS, Kosma V-M, Sillanpää M, Hillamo R, Brunekreef B, Katsouyanni K, Sunyer J, Hirvonen M-R. 2007. Dose and time dependency of inflammatory responses in the mouse lung to urban air coarse, fine, and ultrafine particles from six European cities. INHALATION TOXICOLOGY 19:227-246.

II Happo MS, Hirvonen M-R, Hälinen AI, Jalava PI, Pennanen AS, Sillanpää M, Hillamo R, Salonen RO. 2008. Chemical compositions responsible for inflammation and tissue damage in the mouse lung by coarse and fine particulate samples from contrasting air pollution in Europe. INHALATION TOXICOLOGY 20:1-17.

III Happo MS, Hirvonen M-R, Hälinen AI, Jalava PI, Pennanen AS, Sillanpää M, Hillamo R, Salonen RO. 2009. Seasonal variation in chemical composition of size-segregated urban air particles and the inflammatory activity in the mouse lung. INHALATION TOXICOLOGY (In press.).

IV Happo MS, Salonen RO, Hälinen AI, Jalava PI, Pennanen AS, Cassee F, Gerlofs-Nijland ME, Dormans JAMA, Kosma V-M, Sillanpää M, Hillamo R, Hirvonen M-R. 2009. Inflammation and tissue damage in the mouse lung by single and repeated dosing of urban air coarse and fine particles collected from six European cities. (Submitted).

1 INTRODUCTION

There is a growing awareness that particulate air pollution is responsible for a large variety of adverse health outcomes worldwide. It has been estimated to cause nearly 350 000 premature deaths annually in the European Union countries. Moreover, urban air particles have also been associated with increased hospital admissions, extra medication and millions of lost working days (EU/CAFE, Directive 2008/50).

The most susceptible population groups for these adverse health effects include elderly subjects with chronic cardiorespiratory disease, children and asthmatic subjects of all ages. Particulate induced inflammation in the lungs and systemic circulation has been regarded as the main mechanism exacerbating existing chronic diseases and also inducing new disease cases (Pope and Dockery 2006). On the other hand, reduced particle concentrations have been associated with decreased daily mortality among susceptible population groups (Clancy et al. 2002) and increased life-expectancy of the populations (Laden et al. 2006; Pope et al. 2009).

It has been shown that several characteristics of the particles contribute to their toxicity and inflammatory potency, i.e. their size, shape, surface area, age and chemical composition. Consequently, geographical location, seasonal variation and meteorological conditions may also evoke heterogeneities in the physicochemical characteristics of ambient particles and influence their inflammatory potency.

Only a few previous studies with experimental animals have compared the toxic properties of urban air coarse, fine, and ultrafine particles between each other or addressed the issue of regional, seasonal, and source-related heterogeneities in their toxic properties. These kinds of studies could help to promote the development of better targeted abatement and monitoring strategies, since the current health-based ambient air monitoring and regulation worldwide are mainly based on PM_{10} or $PM_{2.5}$ mass concentrations, assuming all source contributions to be equally toxic.

This dissertation is part of a systematic approach aimed at expanding the present knowledge about the physico-chemical and toxicological characteristics of urban air particles in different size-ranges (Sillanpää et al. 2003, 2005 and 2006; Pennanen et al. 2007, Saarikoski et al. 2008; Saarnio et al. 2008; Jalava et al. 2005, 2007, 2008 and 2009). Six sampling sites and campaign periods across Europe were selected to represent different source environments and seasons of public health interest. In addition, the seasonal variation of particulate properties was measured from samples representing contrasting seasons in Helsinki with regard to temperature, rainfall, and solar radiation.

2 REVIEW OF THE LITERATURE

2.1 Ambient air particulate matter

2.1.1 Particle size-ranges

Urban air particulate matter (PM) is a complex mixture of different sized solid and liquid particles originating from a large variety of anthropogenic and natural sources. The particle size distribution can be achieved by dividing into separate modes based on their aerodynamic diameter: nuclei mode (particle diameter D_p < 0.01μm), Aitken mode (D_p < 0.1 μm), accumulation mode (0.1μm < D_p < 1 μm) and coarse mode (D_p > 1 μm) (HEI, 2002; U.S. EPA 2004). Each of these particles has a distinct source, size range, formation mechanism and chemical composition, as shown in Figure 1.

The nuclei mode particles consist mostly of combustion particles emitted directly into the atmosphere, and from those particles formed in a gas-to-particle conversion (Hinds 1999). The Aitken mode consists of recently formed particles that have grown to a larger size but which are still undergoing active coagulation (U.S. EPA 2004). The lifetime of nuclei and Aitken mode particles is short, because they coagulate with each other and ultimately end up in the accumulation mode. Moreover, the accumulation mode also consists of particles from combustion and photochemically transformed products of volatile organics and oxides of nitrogen (Hinds 1999). Small particles can be removed by diffusion onto surfaces, by falling rain (washout) or by formation of a nucleation surface for cloud droplets (rainout). Coarse mode particles include mechanically generated particles, windblown dust, biogenic material and sea spray. Particles in the accumulation mode coagulate too slowly to achieve the size of coarse-particulate mode, and therefore there is comparatively little mass exchange between these two modes. Therefore, the chemical compositions of these modes are relatively independent of each other (Hinds 1999).

In epidemiological studies, particles are usually divided into ultrafine particles ($PM_{0.1}$, diameter < 0.1 μm), fine particles ($PM_{2.5}$; diameter < 2.5 μm) and coarse thoracic particles ($PM_{10-2.5}$; diameter 2.5-10 μm). Ultrafine particles are commonly measured as a number concentration, while particles in larger size-ranges are measured as gravimetric mass concentration. The comparison of particle properties in different size-ranges is listed in Table 1. Most of the particulate mass worldwide is derived from natural sources, but in urban environments, the particulate mass is usually dominated by anthropogenic sources, such as traffic, combustion processes, road dust and secondary organic and inorganic material. Moreover, geographical

location, seasonal variation and meteorology all make profound contributions to the chemical composition of particulate matter (Sillanpää 2006; HEI 2002; Schwarze et al. 2006).

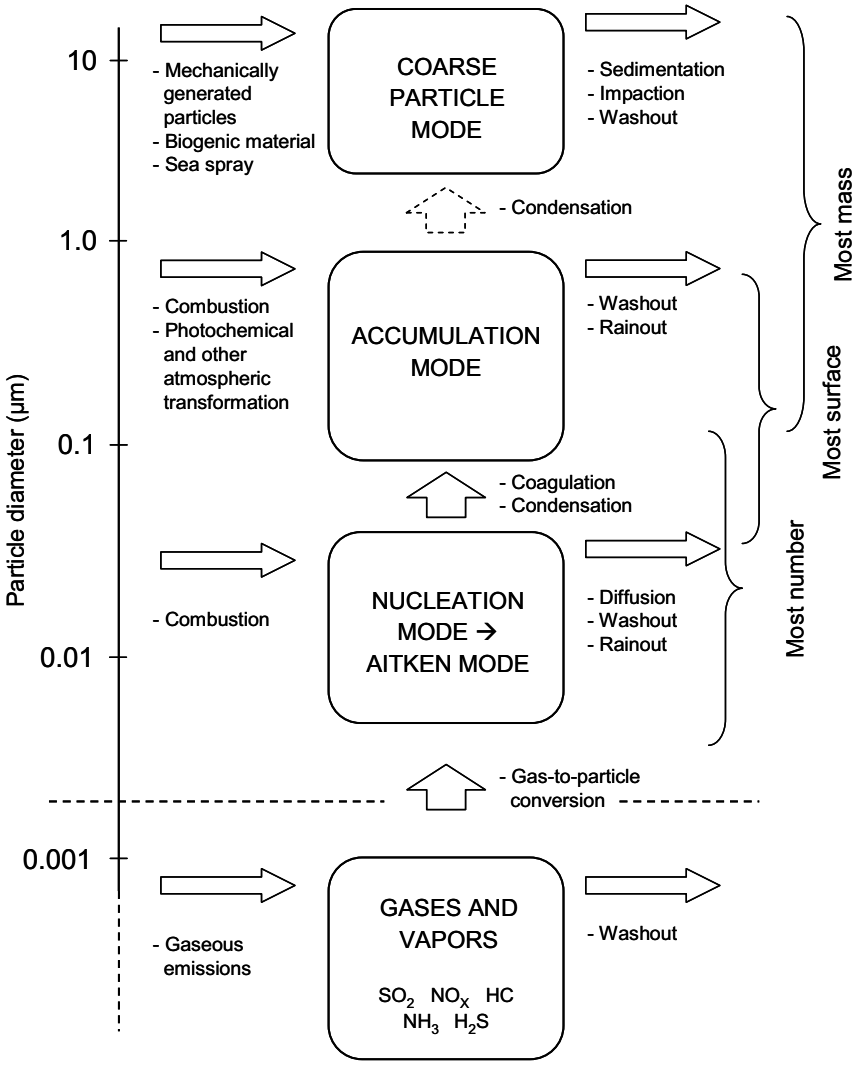


Figure 1. ***Schematic presentation of the main processes of gases and particles in urban atmosphere. Modified from Hinds (1999).***

Table 1. ***Comparison of sources and chemical compositions of urban air particles in different size-ranges used in common health effect studies. (Modified from U.S. EPA 2004).***

	Coarse	Fine	Ultrafine
Sources	Resuspension of industrial and road dust Suspension from disturbed soil Tire, brake pad, and road wear debris Fly ash from uncontrolled combustion Sea spray Biological sources	Combustion of coal and oil Combustion of gas and diesel fuel Biomass burning High temperature processes Atmospheric transformation	Combustion High temperature processes Atmospheric transformation Biological sources
Composition	Nitrates, chlorides and sulfates from HNO_3, HCl and SO_2 reactions with coarse particles Oxides of crustal elements, Zn, Cu Sea salt, NaCl, $CaCO_3$, $CaSO_4$ Pollen fragments, microbes, fungal spores Plant and animal fragments	Sulfate, nitrate, ammonium and hydrogen ions Elemental carbon Organic compounds Trace metals Particle-bound water	Sulfate Elemental carbon Trace metals Organic compounds
Solubility	Largely insoluble and nonhygroscopic	Largely soluble and hygroscopic	Largely soluble
Atmospheric half-life	Minutes to hours	Days to weeks	Minutes to hours
Travel distance	< 1 - 10s of km < 100 - 1000s of km in dust storms	100 - 1000s of km Long range transport	< 1 - 10s of km

In recent years, high volume cascade impactors have been introduced which permit large capacity, size-segregated sampling of urban air particles (Sillanpää et al. 2003, Demokritou et al. 2002, Sioutas et al. 1997). This means that it is possible to collect coarse, fine and even ultrafine particles in sufficient quantities to allow simultaneous chemical analyses and toxicological studies with a variety of *in vivo* and *in vitro* methods.

2.2 Epidemiological background

There is growing scientific evidence that a current level of ambient air particles can cause a wide variety of adverse health effects. The most susceptible population groups to suffer adverse health outcomes include elderly subjects with chronic cardiorespiratory disease, asthmatic subjects of all ages and the children (WHO 2003, Anderson et al. 2004, U.S. EPA 2004). It has been shown in epidemiological studies that urban air thoracic particles (PM_{10}) and in particular fine particles ($PM_{2.5}$) are associated with increased human mortality and morbidity (WHO 2003 and 2005; U.S. EPA 2004). However, in some cases coarse particles have been at least equally potent as fine particles in their ability to cause hospitalization of respiratory patients (Brunekreef & Forsberg 2005). Moreover, some studies have shown that coarse

size-range particles have even demonstrate a stronger association with mortality (Castillejos et al. 2000) and morbidity (Host et al. 2008) than fine particles. In addition to coarse and fine particles, ultrafine particles have been proposed to pose a great risk to human health, because of their high number concentration in urban environments and their ability to penetrate into the blood circulation (Delfino et al. 2005). However, $PM_{2.5}$ has been more strongly associated with cardiorespiratory symptoms than ultrafine particles among elderly subjects (de Hartog et al. 2003). However, it is possible that there may be cumulative health effects from urban particulate pollution, when the duration of elevated exposure to PM_{10} and $PM_{2.5}$ is longer than 24 hours. For instance, the mortality estimates per 10 $\mu g/m^3$ of $PM_{2.5}$ or 20 $\mu g/m^3$ of PM_{10} roughly double within 5 days and can be several times higher if there is chronic exposure for several years, which suggests not only a worsening of existing chronic diseases, but most likely there is also induction of new cardiorespiratory diseases (Pope and Dockery 2006).

The concentration-response relationships of PM_{10} with daily mortality and hospital admissions seem to vary with geographical region and city characteristics (Anderson et al. 2004; Atkinson et al. 2001; WHO 2005; Samoli et al. 2005; Zeka et al. 2006). This kind of heterogeneity can be explained not only by variations in the complex mixture of gaseous and particulate air pollutants, but also the climate and health status of populations (Katsouyanni et al. 2001; Samoli et al. 2005). The relationships have been stronger per unit of mass concentration for cardiovascular mortality and hospital admissions in southern cities of Europe where there is greater photochemical pollution, whereas respiratory mortality has been more prevalent in eastern cities. Moreover, there have been seasonal variations in the mortality estimates for PM_{10} in different regions of the US (Peng et al. 2005). In Europe, mortality and fatal strokes have been associated only during the warm season with PM_{10} in the heavily polluted area of Flanders, Belgium (Nawrot et al. 2007), and with $PM_{2.5}$ in an area of low pollution in Helsinki, Finland (Kettunen et al. 2007).

Recent studies have suggested that it is not only the particulate mass, but also its chemical composition which contribute to the adverse health effects they inflict in susceptible population groups. Source environments that have been considered as being risky to health includes residential heating with wood (Boman et al, 2003; Naeher et al. 2007) and coal (Clancy et al. 2002), living in the vicinity of busy traffic (Hoek et al. 2002; Janssen et al., 2003) and living in vicinity of a poorly controlled steel mill (Ghio 2004).

The chemical constituents of urban air particles that have been associated with a variety of health outcomes include black carbon (Clancy et al. 2002; Hoek et al., 2002; Janssen et al., 2003; Penttinen et al. 2006; Lanki et al. 2006), elemental carbon and organic carbonaceous fraction (Metzger et al. 2004), and transition

metals (Burnett et al. 2000; Ghio 2004). In addition, the SO_4^{2-}-ion has been rather consistently associated with the health outcomes (WHO, 2003; USEPA, 2004), but its causal role has been severely criticized (Schlesinger and Cassee 2003).

2.3 Basic mechanisms of particulate effects

2.3.1 Particle deposition and clearance

Inhaled ambient particles may deposit in the various regions of the respiratory system by several deposition mechanisms, i.e. inertial impaction, sedimentation or diffusion. The extent and location of particle deposition depend on particulate size, density, and shape (Hinds 1999). Moreover, in experimental studies, the animal species used and the exposure techniques also contribute to particle deposition in the respiratory system. The coarse particles are usually deposited in the extrathoracic part of the airways, but to some extent they may also reach the tracheobronchial region and even the alveoli. Most of these particles are fine and ultrafine particles which deposit in the tracheobronchial and alveolar regions of the respiratory tract, whereas the smallest fraction (<10 nm) is mostly deposited in the upper airways (Schwarze et al 2006).

Solid particles deposited into the airways are removed from the surfaces of the respiratory tract by several clearance mechanisms, depending on the region of particle deposition and the solubility of material. In the extrathoracic region, the main clearance mechanisms for poorly soluble particles are mucociliary transport, sneezing, and nose wiping and blowing. In the tracheobronchial region, the most important mechanisms are mucociliary clearance, uptake by macrophages and epithelial cells, and coughing. Moreover, in the alveolar region, uptake by macrophages and epithelial cells can achieve the removal of particles. Ultrafine and the smallest fine particles can even penetrate through the lung tissue and reach the capillary blood vessel and circulating cells. Thereafter, these particles are translocated to other organs, such as liver, spleen, kidneys, heart and brain, where they may be retained (Peters et al. 2006).

Soluble particles usually become rapidly dissolved into fluids covering parts of the respiratory tract and surfaces of alveoli. Thereafter, they are transported through the epithelium into the interstitium, from where they diffuse into the lymph or bloodstream (U.S. EPA 2004).

2.3.2 Inflammatory mechanisms in the lungs

Particulate induced inflammation has been suggested as being the main mechanism of disease exacerbation in both respiratory and cardiovascular subjects (Pope and Dockery 2006). Inflammation occurs when macrophages, other inflammatory cells or epithelial cells have ingested particles and subsequently become activated to release inflammatory mediators. Inflammation also occurs, when particles damage macrophages, activate complement or low-toxicity particles create a large surface area burden (Donaldson and Tran 2002).

Protective responses against airborne pathogens and other micro-organisms or inanimate particles are mediated by early reactions of the innate immunity system followed by later reactions evoked through the adaptive immune system. In the first stage, physical barriers, such as nares, glottis, epithelial layer and mucociliary transport first trap and then sweep particles out of the respiratory tract. If particles manage to evade those first line host defences and penetrate into the lungs, they activate mechanisms of the innate immune system consisting of cellular and biochemical defence mechanisms (Abbas et al. 2007). The mechanisms contributing to host defences are listed in Figure 2.

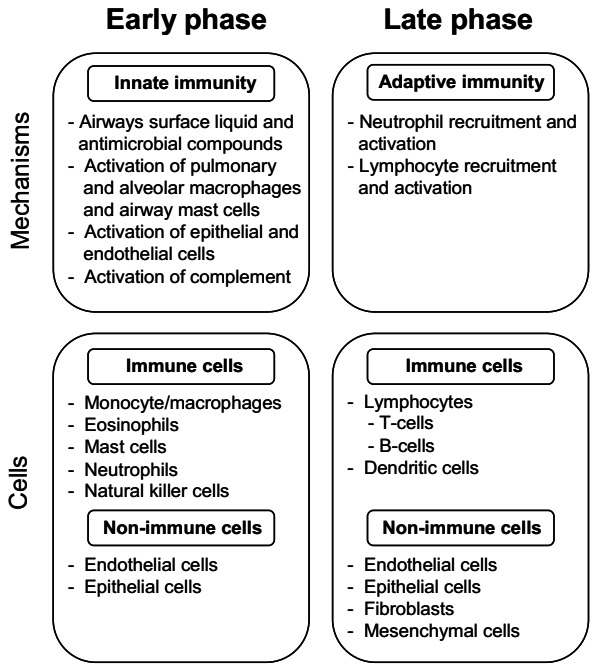


Figure 2. ***The early and late phase host defence mechanisms and cells participating in the inflammatory responses (Modified from Chow et al. 2008).***

Pulmonary epithelium represents a highly effective physical barrier against microbes, since these cells can produce a wide spectrum of antimicrobial and cytotoxic substances. In the present studies, macrophages and neutrophils were anticipated to play the most significant roles in the measured responses in lungs. Their primary roles with some other key cells of innate immunity are listed in Table 2.

Table 2. ***Defence mechanisms of key cells in innate immunity including the secreted inflammatory mediators. (Modified from Chow et al. 2008).***

Immune cell type	Primary functions	Primary or unique inflammatory mediators
Neutrophils	Kill and eliminate invading organisms	Reactive oxygen and nitrogen species Proteolytic enzymes and cationic proteins TNF-α, IL-1ß, IL-6
Macrophages	Immune surveillance Kill and contain invading micro-organisms Removal of particulate matter Antigen presentation	TNF-α, IL-1ß, IL-6 TGF-ß ICAM-1 Reactive oxygen and nitrogen species
Mast cells	"Antennae" of immune response	Granule release TLRs PAF, leukotrienes, and prostaglandins IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, TNF-α, VEGF, TGF-ß, MIP-1α, MCP-1
Dendritic cells	Antigen presentation	TNF-α, IL-1ß
Eosinophils	Allergic response Removal of parasites	Eosinophils' specific granules Cationic proteins Major basic protein Eosinophil peroxidase Eosinophil derived neurotoxin Lipid mediators - leukotriene C_4 and PAF

Macrophages are the primary phagocytic cells, whose main function is to identify, ingest and destroy microbes and activate other inflammatory cells. Neutrophils are the most abundant population of white blood cells and these cells mediate the earliest phases of the inflammatory responses (Abbas et al. 2007). They migrate to sites of infection and tissue damage within a few hours after exposure to microbes or other particulate matter. In contrast, macrophages are present within interstitial tissue, alveolar spaces, and on mucosal surfaces throughout the body (Chow et al. 2008). Macrophages respond to microbes and particles nearly as rapidly as neutrophils, but they survive for a longer period at the areas of inflammation. Therefore, they are the main effector cells at the later stages of the innate immune response. Eosinophils are regarded as effector cells of allergic responses (Chow et al. 2008). Urban air particles may induce eosinophilic inflammation in the lungs of

certain mouse strains with strongly allergic phenotypes (Walters et al. 2001). Lymphocytes are involved in adaptive immune responses. They are the only cells in the body that can specifically recognize and distinguish different antigenic determinants (Abbas et al. 2007). Moreover, the so-called memory lymphocytes ensure a rapid and enhanced response to subsequent exposures to a specific antigen.

The cells of innate and adaptive immunity secrete inflammatory mediators, cytokines and chemokines, which have many functions in these cells. Cytokines are small, soluble proteins that have important roles in the regulation of the inflammatory responses in host defence. They may stimulate, proliferate, differentiate or inhibit specific cell populations. Chemokines are both chemotactic and cellular activating factors for leucocytes (Abbas et al. 2007). They stimulate leucocyte movement and regulate their migration from blood to tissues.

In Studies I-IV of this thesis, two cytokines, TNF-α, IL-6, and one chemokine, KC, were assayed as indicators of inflammation. TNF-α is a proinflammatory cytokine that is mainly secreted by macrophages and T lymphocytes. It is the principal mediator in acute inflammatory responses and is also responsible for a large number of systemic complications encountered in severe infections (Abbas et al. 2007). TNF-α stimulates neutrophil and monocyte recruitment into the sites of infection and activates the cells to destroy microbes and particles. Moreover, it stimulates macrophages to secrete chemokines.

IL-6 is also a proinflammatory cytokine that originates from macrophages, endothelial cells and T cells (Abbas et al. 2007). It is secreted in response to microbes, particles and other cytokines. IL-6 participates in both innate and adaptive immunity. It contributes to the acute phase responses not only by stimulating the synthesis of other inflammatory proteins by hepatocytes, but it also stimulates neutrophil production from its bone marrow progenitors. In adaptive immunity, IL-6 stimulates the growth of differentiated B lymphocytes and acts as a growth factor for neoplastic plasma cells.

KC is mostly secreted by murine keratinocytes, monocytes and macrophages (Ibelgaufts. COPE: http://www.copewithcytokines.de/cope.cgi?key=KC) It is one of the chemokines in the large CXC family. KC and MIP-2 are two recognized candidates for functional murine homologues of the human CXCL8 (formerly IL-8) (Call et al. 2001), which is not found in mice (Rollins 1997). One major role of the CXC chemokines is to attract neutrophils to the local sites of inflammation (Call et al. 2001). KC is a more potent and systemically more extensively distributed chemokine than MIP-2 during the acute phase of inflammation *in vivo* (Call et al. 2001).

2.3.3 Immunosuppression and genotoxicity

In addition to immunostimulatory and proinflammatory effects, it is possible that some mechanisms of the innate or adaptive immune systems are inhibited by constituents of particulate matter. Such immunosuppressive constituents, e.g. PAH compounds, are found in ambient air particles, for example, benzo[a]pyrene has been shown to decrease the levels of proinflammatory cytokines in the lungs (Kong et al. 1994). Accordingly, PAH-rich urban air fine particles from small-scale biomass and coal combustion have been weak inducers of cytokine production but extremely cytotoxic (Jalava et al. 2007 and 2009). In addition, the corresponding ultrafine particles have caused cell cycle arrest in mouse macrophages. Moreover, exposure to concentrated ambient particles (CAPs) gathered from New York City has increased the bacterial burden in the rat lung and decreased neutrophil and cytokine responses to infection in BALF (Zelikoff et al. 2003). On the other hand, combustion particles have been shown to facilitate allergic reactions (Steerenberg et al. 2004).

Several combustion-derived PAH-compounds are well known as being genotoxic *in vitro* and carcinogenic *in vivo* (WHO-IPCS 1998). The genotoxic potential of ambient air particles has been shown be dependent on the quantity of known carcinogenic PAH-compounds bound to the particles (Sevastyanova et al. 2008). The oxy- and nitro-PAHs formed in the atmosphere from the emission products have been demonstrated to form DNA adducts (Lewtas 2007). A high level of DNA-adducts in lung tissue has been associated with an increased risk of cancer (Cheng et al. 2000). It has also been reported that exposure to ambient air $PM_{2.5}$ at even modest levels can induce oxidative DNA damage, which may be related to an increased risk of lung cancer (Sørensen et al. 2003b). Reactive transformation products of PAHs, such as quinones, may be mutagenic by causing reactive oxygen species (ROS) generation and adduct formation (Sørensen et al. 2003; Squadrito et al. 2001). The genotoxicity of ambient air particles was not investigated in this dissertation.

2.3.4 Cytotoxicity and tissue damage

High enough doses of particles can evoke toxic effects in the lungs by several mechanisms. Severe cell injury may lead to necrosis, but also to programmed cell death, i.e. apoptosis. Cytotoxic activity can be measured with biochemical methods. In the present thesis, the pulmonary cytotoxicity induced by ambient air particles was assayed by measuring total protein and LDH concentration in BALF. The leakage of protein into the alveolar space is evidence of interstitial or plasma protein

transudation to the airspaces, which is induced by increased permeability of the alveolar-capillary barrier (Donaldson & Tran 2002; Henderson 1988). This suggests that there is also epithelial injury and tissue damage in the lungs. LDH is cytoplasmic enzyme that occurs extracellularly in BALF only in the presence of damaged cells (Drent et al. 1996), i.e. it is released into the extracellular space after cell lysis or serious damage to the cell membranes.

2.4 Toxicological background

The toxicological mechanisms of adverse health effects of particulate matter have been widely investigated, but there have been only few *in vivo* studies comparing the toxic properties of urban air coarse, fine and ultrafine particles between different sampling campaigns or addressing the issue of regional, seasonal and source-related heterogeneities in the toxic properties of these particles. Moreover, there have been methodological differences between the previous studies e.g. in particulate sampling and extraction, cut-off points of size-ranges, exposure of animals, animal species and strains, particulate doses and time-points of sample collection, which all affect the measured responses. In addition, several *in vivo* studies have used industrially generated artificial particles, e.g. TiO_2 (Dick et al. 2003b; de Haar et al. 2006), carbon black (Gilmour et al. 2004; Li et al. 1999), or standard reference particulate materials representing distinct sources, e.g. diesel engine exhaust (DEP) (Stoeger et al. 2006; Dybdahl et al. 2004; Steerenberg et al. 2003) and residual oil fly ash (ROFA) (Gavett et a. 1999; Kodavanti et al. 2002 and 2000; Steerenberg et al. 2003). However, the results from these studies cannot be directly extrapolated to ambient air particles, because they are a complex mixture originating from a number of different sources. Therefore, toxicological animal and cell studies with validated setups are essential in providing important additional information about the harmful properties of urban air particles. The present review of toxicological studies focuses on studies performed with ambient air particles.

2.4.1 Human exposure to PM

The inflammatory activity of ambient air particles has been studied in the lungs of human volunteers. In these studies, volunteers have been usually exposed to CAPs in exposure chambers or to aqueous extracts of the particulate filter samples instilled through a bronchoscope into lung segments. Exposure to concentrated $PM_{2.5}$ has induced mild inflammation in the lower respiratory tract (Harder et al. 2001; Schaumann et al. 2004; Holgate et al. 2003), as well an increased fibrinogen concentration in blood (Ghio et al. 2000). Exposure of young and healthy volunteers to concentrated $PM_{2.5}$ has not caused any clinically significant cardiorespiratory

effects (Petrovic et al. 2000), whereas acute cardiorespiratory effects, such as changes in autonomic control of heart, inflammatory mediators and clotting factors in circulating blood, have been detected in elderly volunteers (Gong, Jr. et al. 2004). Unexpectedly, volunteers without cardiorespiratory disease have been found to be more vulnerable to fine PM effects in comparison to individuals of similar age with COPD (Gong, Jr. et al. 2004). Moreover, after exposure to diesel exhaust, healthy participants have had small changes in some pulmonary inflammation markers, like the cell differential and cytokine concentrations in BALF, but those changes have not been detected in subjects with mild asthma (Holgate et al. 2003).

It is obvious that not only particulate mass, but also its chemical composition, can influence the potential to cause lung injury. In the study of Ghio and Devlin (2001) with healthy volunteers, bronchoscopically instilled equal masses of PM_{10} samples collected before closure and after reopening of a steel mill had higher inflammatory potency than the corresponding sample taken during its shutdown. Furthermore, it was shown that metal-rich ambient particles could induce influx of monocytes and increased generation of oxidant radicals in human lungs (Schaumann et al. 2004). In particular, the presence of V and Cr in ambient $PM_{2.5}$ has played a major role in the induction of oxidative DNA damage (Sørensen et al. 2005). Coarse particles have been less frequently investigated in controlled human exposures than fine particles. However, it has been shown that inhaled coarse particles can induce neutrophilic inflammation (Alexis et al. 2006) and their biological fraction, that containing especially endotoxins, mediate macrophage responses in the lungs of healthy subjects.

2.4.2 Animal exposure to PM

In toxicological studies in experimental animals, the most often used exposure techniques are aerosol inhalation and intratracheal instillation. These two techniques are known to cause, at least semi-quantitatively, similar inflammatory responses to particulate matter in the rodent lungs (Driscoll et al. 2000; Costa et al. 2006).

Some variability has been noted in the inflammatory potencies of particulate samples in different size-ranges. In general, coarse particles have been the most potent inducers of inflammatory and cytotoxic effects in the rodent lungs, while ultrafine and fine particles have induced only minor pulmonary and systemic effects (Schins et al. 2004; Steerenberg et al. 2004; Kooter, et al. 2006; Gerlofs-Nijland et al. 2007; Gilmour et al. 2007). However, ultrafine particles have occasionally induced strong responses (Dick et al. 2003a; Gilmour et al. 2007). The acute toxicity of fine particles in the rat lungs has been shown to depend on their distinct chemical

composition which is related to their sources i.e. there may be different contributions from vehicles and industry during different seasons (Seagrave et al. 2006).

In previous experiments, particles in different size-ranges have caused neutrophilic inflammation in the lungs and increased total protein, albumin, LDH and cytokine concentration in BALF (Gilmour et al. 2007; Gerlofs-Nijland et al. 2007; Schins et al. 2004) as well as inducing histopathological lesions in lung tissue (Steerenberg et al. 2004; Gerlofs-Nijland et al. 2007). Furthermore, exposure to urban air particles has acted as a kind of adjuvant for antibody production (IgE, IgG_1, IgG_2) in allergic rats (Steerenberg et al. 2004), and evoked vascular responses, such as an increase in the concentrations of fibrinogen in blood (Gerlofs-Nijland et al. 2007) and creatine kinase in the plasma (Gilmour et al. 2007).

The time course of inflammatory response is a major determinant of the measured parameter. However, only a few studies have considered the possibility of different time courses of response parameters in the acute phase of inflammation in rodent lungs (Gerlofs-Nijland et al. 2005; Walters et al. 2001), and none of these studies have examined the time course in relation to particles in more than one size-range. In these studies, the early time points of 3-4 hours have been associated with increased levels of cytokines and chemokines in BALF (Gerlofs-Nijland et al. 2005, Wegesser & Last 2008), whereas the later time points of 12-48 hours have been associated with an increase in the numbers of neutrophils (Gerlofs-Nijland et al. 2005; Wegesser & Last 2008) and eosinophils (Walters et al. 2001).

2.4.3 Cell exposure to PM

In vitro studies are often used for screening of the biological activity of particles. They are also used to clarify mechanisms and pathways behind particulate induced toxic responses. They produce complementary data to epidemiological and clinical studies in human subjects, and to experimental studies in animals. The toxic properties of ambient air particles are usually investigated in macrophage and epithelial cell lines, or primary bronchial and alveolar cells isolated from the rodent or human lungs (Jalava, 2008).

In line with *in vivo* studies, many *in vitro* studies have shown that ambient air coarse particles are more potent inducers of inflammatory responses than fine particles (Hetland et al. 2005; Becker et al. 2003; Monn & Becker 1999; Jalava et al. 2007). However, fine particles have induced a more intense cytokine release from human and mouse macrophages, than ultrafine particles (Becker et al. 2003, Jalava et al. 2007). Particles in the coarse size-range seem to have a higher cytotoxic potential than particles in the smaller size-ranges (Monn & Becker 1999; Jalava et al. 2007).

On the other hand, urban air fine and ultrafine particles rich in combustion derived material have been shown to be potent inducers of apoptosis and cause a distinct cell cycle arrest in the G2/M phase of mouse macrophages (Jalava et al. 2007).

2.5 Heterogeneity in particulate induced toxicity

In addition to particle size and surface area, many geographical, meteorological, chemical composition and source related factors affect the toxic properties of ambient air particulate matter. Only a few studies have been conducted in selected source environments and seasons (Steerenberg et al. 2004, Hetland et al. 2005, Seagrave et al. 2006). In contrast, several toxicological studies have established associations of selected chemical constituents with particulate induced inflammation in *in vivo* or *in vitro* experiments (e.g. Steerenberg et al. 2006, Duvall et al. 2008).

2.5.1 Effects of geographical location and seasonal variation

Particulate samples collected in different geographical locations and seasons have revealed heterogeneities in their abilities to induce toxicological responses both *in vivo* and *in vitro* (Hetland et al. 2005; Jalava et al. 2007; Steerenberg et al. 2004). Fine particulate samples collected in warm and sunny seasons, mostly in the Mediterranean area, have shown higher inflammatory activity in the mouse macrophage cell line than the corresponding samples collected during cool and wet seasons (Jalava et al. 2007). In another study, springtime PM_{10} samples have displayed the highest inflammatory potency in mouse macrophages (Salonen et al. 2004) and in primary macrophages isolated from the rat lung (Hetland et al. 2005). Moreover, particulate samples, regardless of their size-fractions, collected during hot summer months in North Carolina have been more potent inducers of ROS production in human bronchial epithelial cells and alveolar macrophages than the samples collected during colder months. However, there has not appeared to be any similar seasonal predilection in the potency of fine particles to induce cytokine production (Becker et al. 2005). In contrast, both coarse and fine wintertime samples have exhibited the highest adjuvant activity in allergic rats compared to those samples collected in spring and summer (Steerenberg et al. 2004).

2.5.2 Effects of chemical constituents and sources

Enriched metals in urban air particulate samples are associated with enhanced inflammatory responses in several studies. In the Utah Valley, the closure of a poorly controlled steel mill decreased the metal content and toxicity of outdoor PM_{10}

samples in clinical studies (Ghio & Devlin 2001), in animal exposures (Dye et al. 2001) and in cell exposures (Frampton et al. 1999). It has also been shown that the pulmonary effects may not depend exclusively on the total mass of metals, but also on their combination (Dye et al. 2001). Moreover, re-opening of a steel mill in the United Kingdom, increased the Zn, Mn and Cu contents of PM_{10}, and increased its inflammogenic potency in rat lungs (Hutchison et al. 2005). In addition, water-soluble transition metals such as Cu, Mn, Ni, V, Fe(II) and Zn have displayed the highest proinflammatory potency in rat lungs (Rice et al. 2001). It has also been shown that both redox (Cu) and the nonredox (Zn) reactions are involved in the development of lung injury (Prieditis & Adamson 2002).

Relatively few animal studies have examined the inflammatory or toxic potential of ionic compounds in urban air particles. Generally, sulphates and nitrates have shown minimal biological activity even at concentrations well above the commonly measured ambient concentrations (Schlesinger & Cassee 2003). Furthermore, the levels of secondary organic aerosols and sulphate have not correlated with biological responses in the rat lungs (Seagrave et al. 2006). However, the concentrations of secondary inorganic and long-range transport aerosols have exhibited mostly negative or inverse correlations with the inflammatory responses in rats (Steerenberg et al. 2006).

Crustal material is mainly found in coarse particles. There are reports showing that different mineral and metal compositions (Hetland et al., 2000; Becher et al. 2001) and different shapes of mineral particles (Holopainen et al. 2004) may influence the inflammatory and lung damaging activity of urban air coarse particles. Moreover, the amount of crustal material has correlated positively with biological response parameters in mouse and rat lungs (Steerenberg et al. 2006). It has also been shown that proinflammatory responses in the mouse lung are largely driven by the insoluble components of coarse PM samples (Wegesser & Last 2008). Moreover, a dust storm derived fine particulate suspension has been shown to evoke cytotoxic responses in mouse alveolar macrophages *in vitro* (Geng et al. 2006).

A considerable part of the particulate mass consists of organic compounds. However, their role in urban air particulate induced responses is not well understood. Polycyclic aromatic hydrocarbons (PAHs) are known to have genotoxic and carcinogenic properties (WHO-IPCS, 1998), but they may also induce immunomodulatory and cytotoxic effects. Particulate bound PAHs from traffic and incomplete combustion of biomass, coal and heavy fuel oil may act as adjuvants in respiratory allergy (Steerenberg et al. 2006) and mediate both apoptotic and anti-apoptotic signals (Solhaug et al. 2004; Ghanem et al. 2006). Furthermore, the presence of benzo[a]pyrene decreased the levels of proinflammatory cytokines in the BALF of rats (Kong et al. 1994). PAHs in urban air fine particles have also been

associated with immunosuppressive effects and their presence in ultrafine particles has caused cell cycle arrest in the G2/M phase of mouse macrophages (Jalava et al. 2007).

Endotoxins are cell wall lipopolysaccharides of gram-negative bacteria. They are well known for their abilities to induce inflammatory responses both in *in vivo* and *in vitro*. The soil-derived endotoxin content is higher in coarse particles than in fine particles, which is often presented as a potential explanation for the higher potency of coarse particles to induce cytokine and chemokine production (Schwarze et al. 2006). However, in the study of Wegesser & Last (2008), the coarse particle induced inflammatory responses in the mouse lungs were not attributable to the presence of endotoxin.

3 AIMS OF THE STUDY

1. To investigate the dose-relationship and the time course of acute inflammatory responses to size-segregated urban air particulate samples in mouse lungs (I, III).
2. To identify associations of the chemical constituents and the sources of particulate samples with the inflammatory activities (II, III, IV).
3. To investigate the seasonal variation in the inflammatory activity of urban air particulate samples in mouse lungs (I, II, III).
4. To compare inflammatory responses and tissue damage between single- and repeated dosing of urban air coarse and fine particulate samples in mouse lungs (IV).

4 MATERIALS AND METHODS

4.1 Particulate samples (I-IV)

4.1.1 Particle samplers used in field campaigns

Particulate samples were collected with a modified Harvard high volume cascade impactor (HVCI) to enable a high efficiency, representative sampling of particulate mass in different size-ranges (Sillanpää et al. 2003; Pennanen et al. 2007). A schematic cross-section of HVCI is shown in Figure 3. A standard high volume PM_{10}-inlet (Andersen G1200, Village of Cleves, OH, USA) was used to cut-off particles with mean aerodynamic size larger than about 10 μm. The $PM_{10-2.5}$, $PM_{2.5-1}$ and $PM_{1-0.2}$ samples were collected on polyurethane foam (PUF) substrates (PUF; antistatic polyurethane foam 87035K13, McMaster-Carr, New Brunswick, NJ), and the $PM_{0.2}$ samples were collected on glass fibre filters (Munktell MGA, Munktell Filter AB, Grycksbo, Sweden)

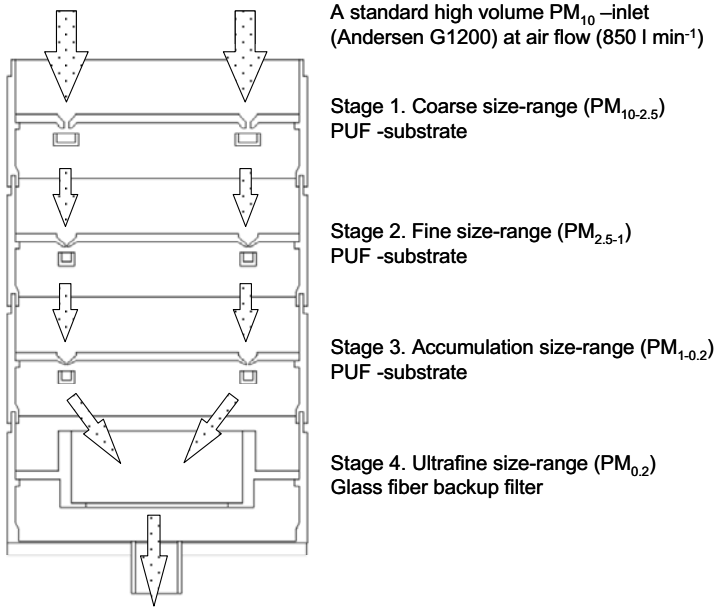


Figure 3. ***Schematic cross-section of the HVCI and overview of the cut-off limits and used substrates.***

4.1.2 Particulate sampling campaigns

The particulate samples were collected in six European cities as presented in Table 3. In Studies I, II and IV, the sampling sites represented urban background air quality and were located in the city centre except for Prague, where the site was in an uptown residential area. They were chosen to represent different source environments and seasons of public health interest. In Study III, the samples were collected in four seasons at the same site that was an urban background station, located 2 km north of downtown Helsinki.

Table 3. ***The sampling campaigns and background information in Studies I-IV, including major emission sources in campaign sites and seasonal features in Study III.***

Sampling site	Study	Sampling period	Season	Major local PM emission sources
Duisburg	I, II, IV	4.10.2002 - 2.11.2002	Autumn	Traffic, metal industry
Prague	I, II, IV	29.11.2002 - 16.1.2003	Winter	Traffic, residential heating with solid fuels
Amsterdam	I, II, IV	24.1.2003 - 13.3.2003	Winter	Traffic, sea
Helsinki	I, II, IV	21.3.2003 - 12.5.2003	Spring	Traffic, harbor, sea
Barcelona	I, II, IV	28.3.2003 - 19.5.2003	Spring	Traffic, harbor, sea, metal industry
Athens	I, II, IV	2.6.2003 - 21.7.2003	Summer	Traffic
				Seasonal feature
Helsinki	III	1.1.2004 - 31.1.2004	Winter	Residential heating, long range transport
Helsinki	III	1.4.2004 - 30.1.2004	Spring	Road dust, pollen
Helsinki	III	1.7.2004 - 31.7.2004	Summer	Pollen
Helsinki	III	1.10.2003 - 31.10.2003	Autumn	Fungal spores

4.2 Sample preparation for chemical and toxicological analysis (I-IV)

The collected particulate samples were transported frozen to the National Public Health Institute, Kuopio, Finland, where they were stored at -20°C. Before sampling, all the PUF substrates and glass fibre filters were washed with methanol, dried in +50°C for 24 hours and weighed using an analytical balance (Mettler

Toledo AG 285, Mettler Instrumente AG, Zurich, Switzerland). The sampled PUF strips or quarters of glass fibre filter were weighed and subsequently extracted with methanol (J. T. Baker HPLC grade, Deventer, The Netherlands) for 2 x 30 min in a water bath sonicator (FinnSonic m20, Finnsonic Oy, Lahti, Finland) at +20°C. The methanol extracts from the particulate-loaded substrates of each city (I, II and IV) or season (III) were pooled according to size-range, and excess methanol was evaporated at +35 °C with a rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany) attached to a vacuum pump (Vacuubrand CVC 2000, Wertheim, Germany) set at 150mbar. The methanol suspension containing $PM_{0.2}$ particles was filtered (Schleicher & Schuell FP 30/0.2 CA-S filter, pore size 0.2 µm, Dassel, Germany) to remove glass fibres derived from filters. The concentrated suspension was divided into 10-ml glass tubes as the defined amount of particulate mass and dried under nitrogen (99.5%) flow. The resultant dried samples were stored at -20°C prior to the subsequent animal exposures and chemical analyses. A procedure that was similar to the size-segregated particulate samples was used in the preparation of the corresponding pooled blanks (Jalava et al. 2006).

The mean extraction efficiency of particulate matter from the PUF substrates was 85% (range 61-99%), with a tendency toward a higher efficiency with samples in smaller size ranges. The extraction efficiency could not be measured for the $PM_{0.2}$ particles collected on glass fibre filters.

In the animal exposures, the dry particulate samples and blanks were thawed and stabilized to room conditions. Thereafter, they were suspended into pathogen-free water (Sigma, W1503) at a concentration of 10 mg/ml and sonicated for 30 min in a water-bath sonicator (Finnsonic m03, Finnsonic Oy, Lahti, Finland). The suspension was diluted in pathogen-free water to obtain final concentrations of 0.5, 1.5 and 5 mg/ml to be used in animal exposures. The blank samples were diluted in an equal volume of pathogen-free water to ensure that the vehicle of particulate suspension and possible impurities in methanol extraction were not sources of toxic activity. Pathogen-free, endotoxin tested, sterile filtered water was used as the vehicle, because the aqueous suspensions of particulate samples contained substantial amounts of cations and anions (e.g. sea salt) and no modification of the chemical composition of the samples was desired.

4.3 Chemical analyses and source characterization of particulate samples (II-IV)

The list of methods used in chemical analyses and the chemical constituents analysed from the particulate samples are shown in Table 4.

Table 4. ***Methods used in chemical analyses and chemical constituents analysed from particulate samples.***

Method	Abbreviation	Analysed constituents
Ion chromatography	IC[1,2]	Anions: Cl^-, NO_3^-, SO_4^{2-}, succinate, malonate, oxalate Cations: Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}
Inductively coupled plasma mass spectroscopy	ICP/MS[1,2]	Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V, Zn
Energy dispersive X-ray fluoresence	ED-XRF[2]	Al, Ca, Cl, Cu, Fe, K, Mn, Ni, Pb, Si, V, Zn
High-performance liquid chromatography with mass spectrometry	HPLC/MS[2]	Sum of levoglucosan, galactosan, mannosan (ΣMA)
Thermal optical carbon analyser	TOA[2]	EC, OC
Gas chromatograph mass spectrometry with selected ion monitoring	GCMS-SIM[1]	Total of 32 PAH-compouds
Limulus amebocyte lysate-assay	LAL	Endotoxin

[1]Analysed from HVCI-samples.
[2]Analysed from VI-samples.
*The PAH compounds in the EU-directive (Directive 2004/107/EC) were used in the analysis of associations with toxic responses.
Note: All analysis methods and procedures are described in detail in Sillanpää et al. 2005 and 2006, excluding LAL in Hollander et al. 1993.

A chemical mass closure method was used for characterization of different sources and the major components of particulate matter in the sampling campaigns (Sillanpää et al. 2006). The components and the calculation formulas for chemical mass closure are presented in Table 5.

4.4 Animals (I-IV)

Pathogen-free male C57Bl/6J mice 8- to 9 week-old (weight 19-31g) were used in all the studies. The animals were obtained from the breeding colony of the National Public Health Institute, Department of Environmental Health, Kuopio, Finland. They were transferred from a barrier unit to a conventional animal room 2 weeks before the experiments. After a one-week acclimatization period, the animals were transferred into metal cages and they were housed singly on aspen wood chips (FinnTapvei, Finland). The mice received water and R36 maintenance diet (Lactamin, Stockholm, Sweden) *ad libitum*. The animals were kept on a 12-hour

light/dark cycle (7 a.m. to 7 p.m.) at room temperature (21 °C) and relative humidity of 46 ± 5% (mean ± SD).

Table 5. ***Calculation of the components of chemical mass closure in the HVCI fine ($PM_{2.5-0.2}$) and coarse ($PM_{10-2.5}$) particulate samples.***

Parameter	Abbreviation	Formula
Non-sea-salt sulphate	nss-SO_4^{2-}	[Nss-SO_4^{2-}] = [SO_4^{2-}] - 0.246 x [Na^+]
Nitrate[a]	NO_3^-	
Ammonium[a]	NH_4^+	
Sea salt[a]	SS	[SS] = 3.248 x [Na^+]
Water-soluble soil[a]	WSS	[WSS] = [Fe_2O_3] + [Al_2O_3] + [CaO] + [K_2O]
Water-insoluble soil[b]	WIS	[WIS] = [Fe_2O_3] + [SiO_2] + [Al_2O_3] + [CaO] + [$CaCO_3$] + [K2O] - [WSS]
Other elements[a]	OE	[OE] = [As]+[Cd]+[Co]+[Cr]+[Cu]+[Ni]+[V]+[Mn]+[Pb]+[Zn]
Elemental carbon[c]	EC	
Particulate organic matter[c]	POM	[POM] = 1.4 * OC
Unidentified matter	UM	[UM] = [gravimetric PM_x] - [sum of identified components of PM_x]

[a]Constituent concentrations from the IC and ICP-MS analysis of the HVCI-$PM_{2.5-0.2}$ and HVCI-$PM_{10-2.5}$ samples (Pennanen et al. 2007).

[b]The total concentrations of Fe, Si, Al, Ca and K in the HVCI-$PM_{10-2.5}$ and HVCI-$PM_{2.5-0.2}$ samples were estimated with the help of simultaneous virtual impactor data (VI) (Sillanpää et al. 2006).

[c]The relative (percentage) values were taken directly from the VI data of Sillanpää et al. (2006) on EC and POM.

4.5 Experimental design for animal studies (I-IV)

A well-established mouse model (Jussila et al., 2001) has been used due to a close resemblance of the immunology of this species to humans and a low consumption of particulate mass in intratracheal instillations. The Ethical Committee of the University of Kuopio for Animal Experiments has approved all present study plans.

All the tested samples and studies are summarized in Table 6. Study I contained both a dose-response screening of the particulate samples and a time-course investigation of the biochemical and cytological parameters used in recording the acute inflammatory response in BALF. As control groups, we used 1) untreated mice, 2) mice intratracheally treated with pathogen-free water (Sigma), 3) mice exposed to solution extracted from blank filters (negative controls) and 4) mice exposed to Ottawa dust (EHC-93; positive controls - data not shown) (Vincent et al. 1997).

In Studies II and III, the most optimal time points of response recording observed in Study I were used for measurement of inflammatory markers in BALF: 4 hours for cytokines and 12 hours for cell number and total protein concentration in BALF. Subsequently, the associations of chemical constituents with the measured inflammatory responses were analysed.

In Study IV, the mice were intratracheally instilled with a single dose or repeated doses of particulate matter. In the repeated dosing, the animals were exposed to a fixed particle dose on days 1, 3 and 6. The lungs of exposed mice were lavaged 24 hour after the single dose or the last repeated dosing. Tissue samples for histopathological examination were also collected using the same time schedule.

Table 6. ***Particulate sampling sites, size-ranges, doses as well as time-points of response recording and detected markers of inflammation and tissue damage in the four studies. $PM_{2.5-0.2}$ samples are derived from pooling of $PM_{2.5-1}$ and $PM_{1-0.2}$ size-range samples.***

Study	Sampling sites	Size ranges	Doses (mg/kg)	Time-points (h)	Markers
I	Duisburg	$PM_{10-2.5}$	1	24	Total cell number,
	Prague	$PM_{2.5-0.2}$	3		Cell differentials,
	Amsterdam	$PM_{0.2}$	10	4	Total protein concentration,
	Helsinki			12	LDH, TNF-α, IL-6, KC
	Barcelona			24	
	Athens				
II	Duisburg	$PM_{10-2.5}$	10	4	Total cell number,
	Prague	$PM_{2.5-0.2}$		12	Total protein concentration,
	Amsterdam				TNF-α, IL-6, KC
	Helsinki				
	Barcelona				
	Athens				
III	Helsinki Winter	$PM_{10-2.5}$	10	4	Total cell number,
	Helsinki Spring	$PM_{2.5-1}$		12	Total protein concentration,
	Helsinki Summer	$PM_{1-0.2}$			TNF-α, IL-6, KC
	Helsinki Autumn	$PM_{0.2}$			
IV	Duisburg	$PM_{10-2.5}$	10	24	Total cell number,
	Prague	$PM_{2.5-0.2}$	3x 10[a]	24[b]	Cell differentials,
	Amsterdam				Total protein concentration,
	Helsinki				LDH, TNF-α, IL-6, KC
	Barcelona				Histopathology
	Athens				

[a]Repeated dose

[b]24h after the last given dose

4.6 Intratracheal instillation (I-IV)

The mice were anesthetized with vaporized 4.5% sevoflurane (Sevorane, Abbott, IL) and positioned in a 66° upward bent position with the incisors placed on a thin wire. The instillation of particulate suspension was performed under visual control, while the tongue was pulled out with forceps to prevent the mouse from swallowing. The samples were delivered onto the vocal folds with a Finn pipette tip (Finntip 200 Ext, Thermo Electron Oy, Vantaa, Finland). Thereafter, the nostrils were covered forcing the mouse to inspire the instilled particle suspension.

4.7 Bronchoalveolar lavage and analyses from BALF (I-IV)

At the defined time point, the mice were anesthetized with pentobarbital (60 mg/kg) and exsanguinated by cardiac puncture. The lungs were perfused with sterile saline. Thereafter, the trachea was cannulated with polyethylene tubing and the lungs were lavaged with two portions of sterile saline (30 ml/kg), three times each. These two portions of BALF were combined and kept on ice. Cells were separated from the BALF by centrifugation (500g, 10 min) and the supernatant was removed for separate analyses. LDH and total protein concentrations were analysed from fresh supernatant, while the remaining portion was frozen and stored (-80 °C) for subsequent cytokine analyses.

4.7.1 Cell count

The cell pellet was resuspended into 220µl of sterile saline for cell counting. Total cell number was microscopically counted from each pellet sample by using a Bürker chamber and the trypan blue exclusion method.

4.7.2 Cell differential

The remaining cell suspension was used for cell differential determination by cytospin (210 µl, 500 rpm, 8 min; Megafuge, Heraus Instruments, Germany). Slides were fixed with May-Grünwald-Giemsa dye. For cell differential, 100 cells were counted from 3 different views of the slide and the mean percentages for each cell type were calculated.

4.7.3 Biochemical analyses

Lactate dehydrogenase (LDH) and total protein concentration were analysed from fresh supernatants. LDH was analysed by using a cytotoxicity detection kit (Roche Diagnostics GmbH, Germany) with minor modifications as earlier described by Jussila et al. (2001). Total protein concentration was analysed by a modified DC protein assay (Bio-Rad, Hercules, CA) as earlier described in detail by Jussila et al. (2001).

4.7.4 Immunochemical analyses

Tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and keratinocyte-derived chemokine (KC) were analysed from the BALF supernatant. All immunochemical analyses were made with commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions as earlier described in detail by Jalava et al. (2005).

4.8 Histopathological analyses (IV)

Lungs of the animals selected for histopathological examination were removed and filled with 10% phosphate buffered formalin, which was also used in the preservation of the tissue samples prior to subsequent histopathological examination. Thereafter, they were trimmed and embedded in paraffin and cut at 5 µm sections. After cutting, the tissues were stained with hematoxylin and eosin. Both the left and right lungs were examined under a light microscope. Lesions were semi-quantitatively scored as follows: - absent, 1 = minimal, 2 = slight, 3 = moderate, 4 = marked and 5 = strong.

4.9 Statistical methods (I-IV)

All the statistical methods used in Studies I – IV are listed in Table 7. Statistical analyses were made using SPSS software versions 13-16.0.2 and the power fit function of Microsoft Office Excel 2003 SP2 in Study II.

Table 7. ***Statistical methods used in Studies I - IV.***

Study	Analysis	Method
I	Equality of variances	Levene's test
	Dose response and time course	Analysis of variance (ANOVA) Dunnett's test Kruskal-Wallis
	Differences between sampling sites	Tukey's HSD Dunnett's C
II	Relationship between the variables	Spearman's (ρ) rank
	Trends between KC and selected source indicators	Power fit function
III	Equality of variances	Levene's test
	Time course	Analysis of variance (ANOVA) Dunnett's test Kruskal-Wallis
	Differences between seasons	Tukey's HSD Dunnett's C
	Relationship between the variables	Spearman's (ρ) rank
IV	Equality of variances	Levene's test
	Time course	Analysis of variance (ANOVA) Dunnett's test Kruskal-Wallis
	Differences between sampling sites	Tukey's HSD Dunnett's C
	Differences between single- and repeated dosing	2-tailed Mann-Whitney test
	Relationship between the variables	Spearman's (ρ) rank
	Differences in histopathological changes	Pearson Chi-square test Fisher's exact test

5 RESULTS

5.1 Effects of particle dosing

5.1.1 Single dosing (I)

The dose-dependencies of the increases in total cell number in BALF at 24 h after exposure to the $PM_{10-2.5}$ and $PM_{2.5-0.2}$ samples from six European cities are shown in Figure 4. All the $PM_{10-2.5}$ samples showed clear dose-dependent responses at the doses of 1, 3 and 10 mg/kg, while only two $PM_{2.5-0.2}$ samples out of six induced clear increases in the BALF total cell number at the largest mass dose.

The response patterns in the BALF total protein and cytokine concentrations were rather similar to those in total cell number. All the detected cytokine responses in dose-dependency study were relatively small. The $PM_{0.2}$ samples induced only negligible inconsistent responses without any observable dose-dependency (I).

There were different time-course profiles in the detected inflammatory responses to the urban air particulate samples. Total cell number (Figure 5) and protein concentration (I) in BALF most often peaked 12 hours after intratracheal instillation, whereas the highest cytokine concentrations (TNF-α, IL-6, KC) were consistently measured already at 4 hours after the exposure (Figure 6). These time-course profiles applied to both the $PM_{10-2.5}$ and the $PM_{2.5-0.2}$ samples, while the responses to the $PM_{0.2}$ samples were minimal.

The total cell number was not increased at 4 hours by any of the samples. In contrast, the highest total cell numbers were detected at 12 hours with all the $PM_{10-2.5}$ samples and with most of the $PM_{2.5-0.2}$ samples. At the latest 24-hour time point, the cell numbers were still increased from the corresponding control level, especially with the $PM_{10-2.5}$ samples. Cell differential analysis revealed that the increase in total cell number at 12 hours was mainly due to a large increase in the numbers of neutrophils in BALF. A slight increase in the number of lymphoplasmacytic cells was also detected with some of the fine and coarse particulate samples.

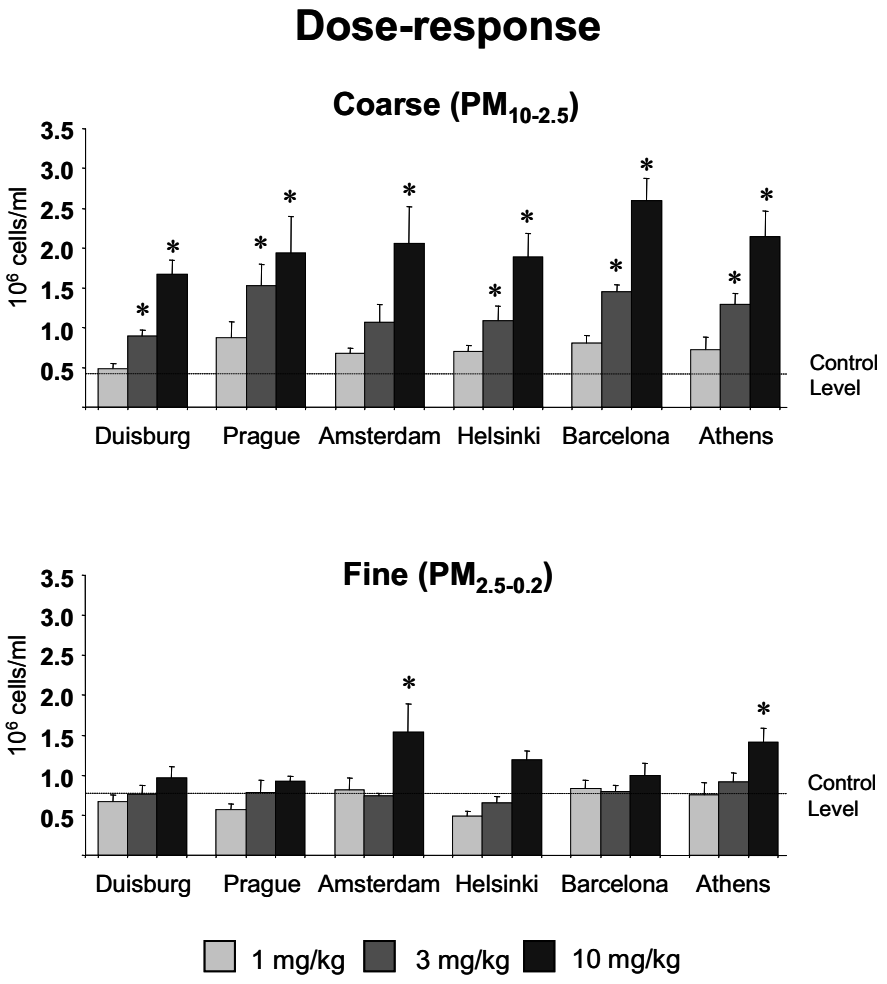


Figure 4. ***Total cell number in BALF from healthy C57Bl/6J mice at 24 h after intratracheal exposure to a single dose (1, 3, or 10 mg/kg) of particulate samples in two size ranges. Control levels are from exposure to a corresponding blank sample. Each bar shows mean ± SE (n = 5-8), Asterisk indicates statistically significant difference from the control (Dunnett, p < 0.05).***

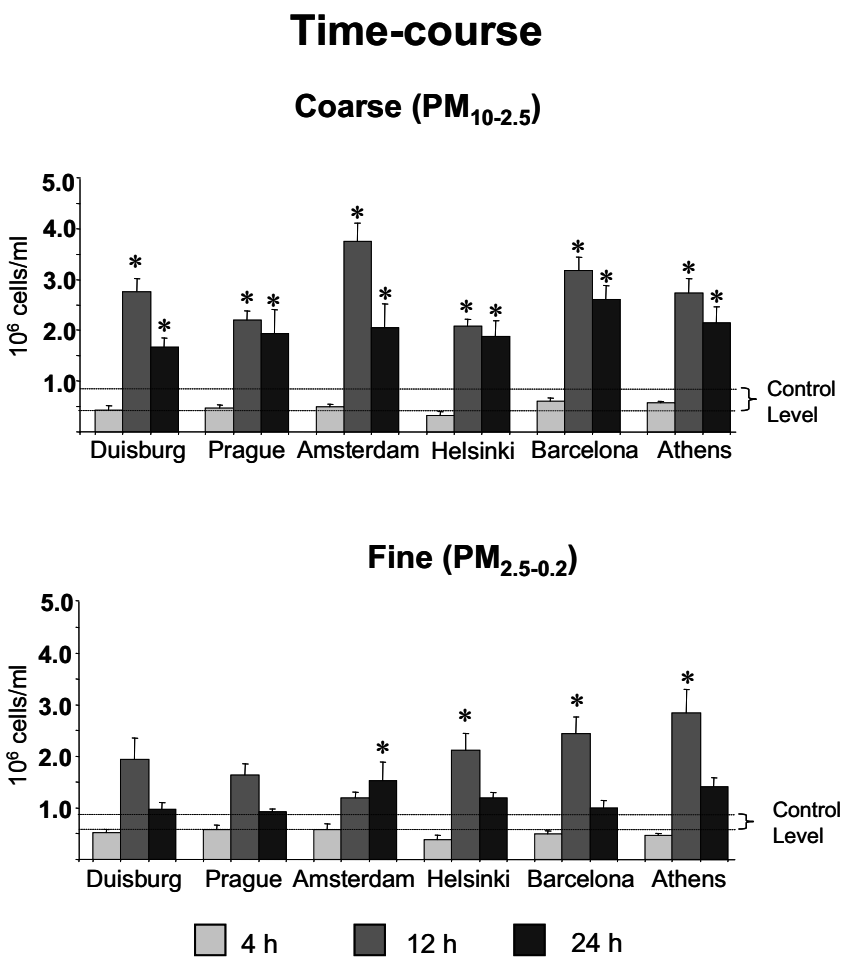


Figure 5. ***Total cell number in BALF from healthy C57Bl/6J mice at 4, 12, and 24 h after intratracheal exposure to a single dose (10 mg/kg) of particulate samples in two size ranges or to a corresponding blank sample. Each bar shows mean ± SE (n = 5-8), Asterisk indicates statistically significant difference from the control (Dunnett, p < 0.05).***

None of the particulate samples increased significantly total protein concentration at 4 hours after exposure. The largest responses were measured at 12 hours with most of the samples, as only three out of 12 particulate samples induced their largest response at 24 hours (I).

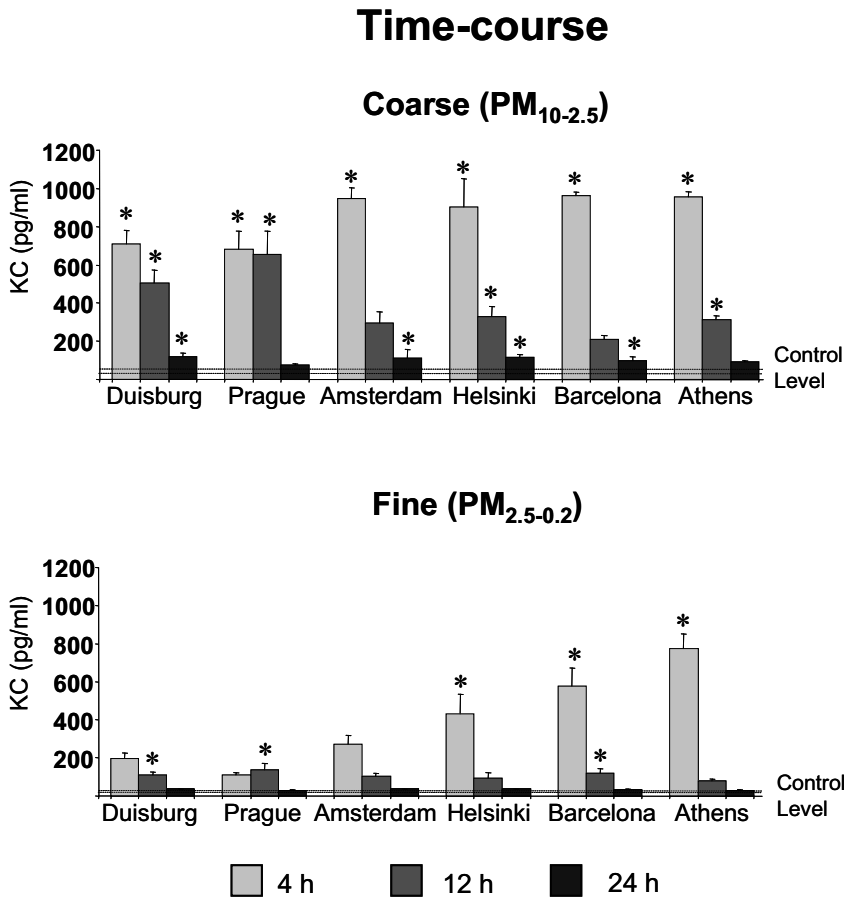


Figure 6. ***KC concentration in BALF from healthy C57Bl/6J mice at 4, 12, and 24 h after intratracheal exposure to a single dose (10 mg/kg) of particulate samples in two size ranges. Control levels are from exposure to a corresponding blank sample. Each bar shows mean ± SE (n = 5-8), Asterisk indicates statistically significant difference from the control (Dunnett, p < 0.05).***

The highest detected cytokine concentrations in BALF were measured at the earliest 4 h time point. All of the $PM_{10-2.5}$ samples evoked large increases in the cytokine concentration in BALF, but $PM_{2.5-0.2}$ samples displayed greater heterogeneity in their inflammatory potency. At 12 hours after exposure, most of the cytokine responses were much smaller than those at 4 hours. Only minimal cytokine responses were seen at the latest time point of 24 hours.

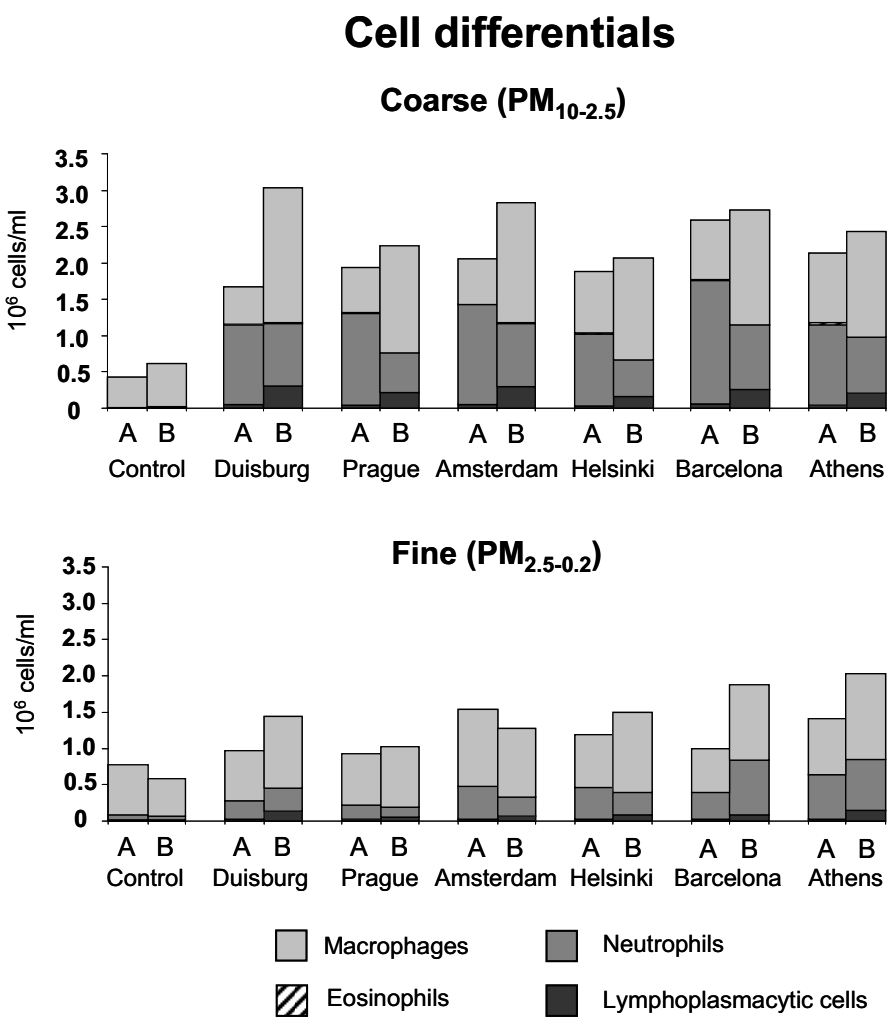


Figure 7. ***Cell differentials in BALF from healthy C57Bl/6J mice (n = 5-6) after intratracheal exposure to a single dose (A) or after repeated dosing (B) of particulate samples (10 mg/kg) in two size-ranges. Control mice were exposed to a corresponding blank sample.***

5.1.2 Repeated dosing (IV)

There were no major differences in the total cell numbers in BALF between the single dose and repeated dosing of particulate samples in mice (Figure 7). However, the numbers of macrophages, neutrophils and lymphoplasmacytic cells were often

significantly increased after repeated dosing of both the $PM_{10-2.5}$ and $PM_{2.5-0.2}$ samples. In many cases, the number of neutrophils in BALF was lower after repeated dosing than after a single dose of the corresponding sample.

The single dose and the repeated dosing of particulate samples evoked rather similar increases in the total protein concentration in BALF (IV). The single dose exposure induced inconsistent LDH responses (data not shown). In contrast, after the repeated dosing $PM_{10-2.5}$ samples increased significantly the LDH concentration in BALF, but the $PM_{2.5-0.2}$ samples showed no changes from the corresponding control level (data not shown).

Single dose exposure increased cytokine concentrations in BALF more than repeated dosing with most of the particulate samples. With the latter treatment, the cytokine concentrations were only slightly higher than control levels. The $PM_{10-2.5}$ samples were more potent inducers of cytokine production than the $PM_{2.5-0.2}$ samples (IV).

Inflammatory lesions in the mice lungs were more extensive after repeated dosing of both the $PM_{10-2.5}$ and the $PM_{2.5-0.2}$ samples, when compared to the single dose of the corresponding samples (IV). In general, the $PM_{10-2.5}$ samples evoked slightly more extensive lesions than the corresponding $PM_{2.5-0.2}$ samples.

5.2 Differences in pulmonary responses

5.2.1 Sampling sites (I, IV)

In general, the $PM_{10-2.5}$ samples from all sampling sites had higher inflammatory activities than the corresponding samples in the smaller size-ranges. However, the $PM_{2.5-0.2}$ samples showed a larger heterogeneity in their inflammatory activities than the $PM_{10-2.5}$ samples, when all BALF parameters were measured at their most optimal time-points.

In the single dose study (I), $PM_{2.5-0.2}$ samples from the Mediterranean sampling campaigns, Barcelona and Athens, evoked the highest inflammatory responses in the mouse lungs compared to Duisburg, Prague and Amsterdam. There were no differences in the negligible responses to the $PM_{0.2}$ samples between sampling campaigns (I).

After repeated dosing of the $PM_{10-2.5}$ and $PM_{2.5-0.2}$ samples, the response patterns in the total cell number in BALF resembled those seen at 12 hours after the single dose exposure. The most extensive pathological lesions in the mouse lungs were caused by particulate samples from Helsinki, Barcelona and Athens in both size-ranges. The

heterogeneity in the severity of inflammatory lesions was larger with the $PM_{2.5-0.2}$ samples (IV).

5.2.2 Seasons (III)

There was a clear seasonal variation in the inflammatory activities of particulate samples collected in Helsinki. This was seen in several BALF parameters such as total cell number and the cytokine concentrations.

The highest inflammatory activities were measured with the $PM_{10-2.5}$ and $PM_{2.5-1}$ samples of spring, summer and autumn (Figure 8). The corresponding winter samples had clearly the lowest inflammatory activity in most of the response parameters. The samples in the smaller $PM_{1-0.2}$ and $PM_{0.2}$ size-ranges showed only slight or negligible inflammatory activity in BALF.

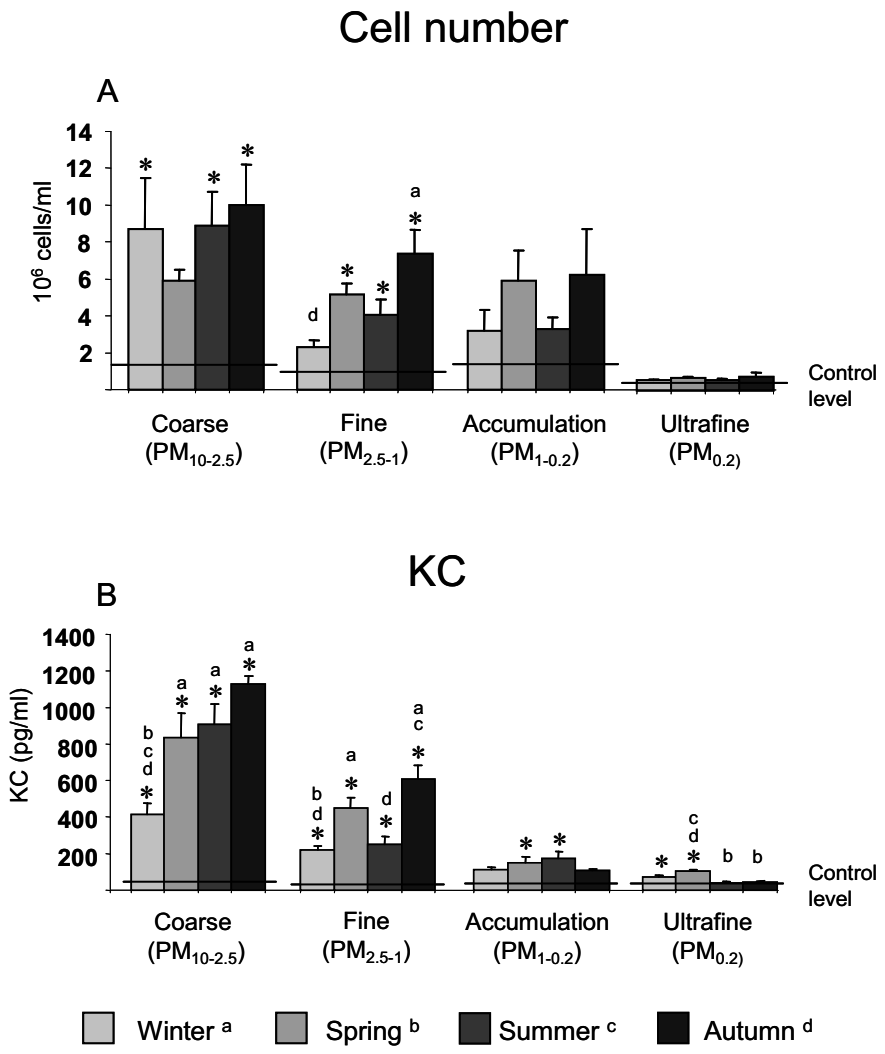


Figure 8. ***Total cell number in BALF from healthy C57Bl/6J mice at 12 h (A) and KC concentration at 4 h (B) after intratracheal instillation of a single dose (10 mg/kg) of particulate samples in four size ranges. Control levels are from exposure to the corresponding blank samples. Each bar shows mean ± SE (n = 5-6, except for $PM_{0.2}$ n = 2-3). An asterisk indicates statistically significant differences from the blank control (Dunnett's C test, p < 0.05). Letters a, b, c and d indicate statistically significant differences from the responses to other seasonal samples (Tukey-HSD test, p < 0.05).***

5.3 Relative inflammatory activity induced by particulate samples (I, III)

Table 8 shows the relative inflammatory activities induced by the particulate samples according to the maximal responses (I) or to responses at the most optimal time-points (III) for the response parameters in BALF. The Barcelona and Athens campaigns displayed the highest $PM_{10-2.5}$ mass concentration in urban air and high inflammatory activities of the corresponding particulate samples per unit of mass. Consequently, the particulate material in the $PM_{10-2.5}$ size-range collected from these cities had by far the highest inflammatory potential per cubic meter of air as assessed on the basis of all response parameters. In the seasonal campaigns in Helsinki, the springtime road dust contributed to the high $PM_{10-2.5}$ concentration and had also clearly higher inflammatory potential per cubic meter of urban air, when compared to other seasons.

In $PM_{2.5-0.2}$, particulate material collected in the Athens campaign had the highest inflammatory potential per cubic meter of urban air, followed by the Barcelona campaign in BALF cytokines. Particulate materials gathered in Duisburg, Prague and Amsterdam had most often the lowest inflammatory activities per unit of mass, but due to the highest $PM_{2.5-0.2}$ concentrations in urban air they did not have the lowest overall inflammatory potential (I).

A low inflammatory potential was also observed for the wintertime $PM_{2.5-1}$ material in Helsinki. The spring sample had the highest inflammatory activity per unit of $PM_{2.5-1}$ mass and also the highest overall inflammatory potential. The $PM_{1-0.2}$ and $PM_{0.2}$ materials of spring and winter were assessed to have the highest inflammatory potential of all seasons per cubic meter of urban air.

5.4 Chemical constituents responsible for inflammatory activity in the mouse lung (II, III, IV)

The correlation coefficients between selected chemical constituents and inflammatory response markers for the $PM_{10-2.5}$ and $PM_{2.5-0.2}$ samples from six European cities are shown in Figures 9 and 10. There was a larger number of chemical constituents and sources associated with the inflammatory activity of the $PM_{2.5-0.2}$ samples in the mouse lung than with the $PM_{10-2.5}$ samples. Moreover, the chemical compositions of the $PM_{2.5-0.2}$ samples were more heterogenic than those of the $PM_{10-2.5}$ samples (II).

Table 8. *Means of size-segregated particulate mass concentrations in urban air during the sampling campaigns and the relative inflammatory activities of the particulate samples in the different size-ranges.*

Study	Size range	Sample	Mean of PM mass (μg/m^3)	Relative response per μg of PM mass | per m^3 of air: Cell number		Protein		TNF-alpha		IL-6		KC	
I, II	$PM_{10-2.5}$	Duisburg	7.6	1.3	1.6	**1.5**	1.6	1.0	1.0	1.8	2.4	1.0	1.3
I, II		Prague	5.9	1.1	1.0	1.2	1.0	1.8	1.4	1.0	1.0	1.0	1.0
I, II		Amsterdam	9.8	**1.8**	2.8	1.2	1.7	1.9	2.5	1.5	2.5	**1.4**	2.3
I, II		Helsinki	12.8	1.0	2.1	1.0	1.8	1.3	2.1	1.6	3.5	1.3	2.9
I, II		Barcelona	22.9	1.5	5.6	1.3	4.2	**2.9**	8.8	1.7	6.6	**1.4**	5.5
I, II		Athens	**29.6**	1.3	**6.2**	1.1	**4.5**	2.3	**8.9**	**1.9**	**9.7**	**1.4**	**7.1**
I, II	$PM_{2.5-0.2}$	Duisburg	15.8	1.3	1.7	1.1	1.8	2.0	1.2	2.7	2.1	1.4	1.0
I, II		Prague	**25.1**	1.1	2.3	1.1	**2.8**	1.0	1.0	1.0	1.2	1.0	1.1
I, II		Amsterdam	22.8	1.0	2.0	1.0	2.4	4.6	4.2	1.6	1.8	2.0	2.0
I, II		Helsinki	8.3	1.4	1.0	1.2	1.0	3.1	1.0	2.5	1.0	3.1	1.1
I, II		Barcelona	14.3	1.6	2.0	1.3	2.0	6.6	3.7	3.9	2.7	4.2	2.6
I, II		Athens	18.9	**1.8**	**3.0**	**1.4**	**2.8**	**10.0**	**7.5**	**5.8**	**5.4**	**5.6**	**4.7**
III	$PM_{10-2.5}$	Winter	2.5	1.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
III		Spring	**15.1**	1.0	**4.0**	1.1	**6.8**	4.9	**29.4**	3.3	**19.7**	2.0	**12.0**
III		Summer	4.4	1.5	1.8	1.2	2.1	4.3	7.5	3.4	5.9	2.2	3.8
III		Autumn	4.4	**1.7**	2.0	**1.6**	2.8	**6.6**	11.5	**4.8**	8.5	**2.7**	4.8
III	$PM_{2.5-1}$	Winter	3.0	1.0	1.1	1.0	1.6	1.0	1.2	1.0	2.0	1.0	1.7
III		Spring	**3.1**	2.3	**2.5**	1.5	**2.4**	4.6	**5.5**	2.0	**3.9**	2.1	**3.6**
III		Summer	1.5	1.8	1.0	1.2	1.0	1.7	1.0	1.0	1.0	1.1	1.0
III		Autumn	1.4	**3.2**	1.7	**1.6**	1.2	**4.8**	2.7	**2.1**	1.9	**2.8**	2.3
III	$PM_{1-0.2}$	Winter	**7.4**	1.0	1.7	1.0	**2.0**	1.0	1.6	1.0	1.1	1.0	2.0
III		Spring	5.8	1.8	**2.5**	**1.1**	1.8	**1.5**	**2.0**	**1.8**	**1.6**	1.4	**2.2**
III		Summer	4.2	1.0	1.0	1.0	1.1	1.1	1.0	1.5	1.0	**1.6**	1.8
III		Autumn	3.7	**2.0**	1.7	1.0	1.0	**1.5**	1.2	**1.8**	1.0	1.0	1.0
III	$PM_{0.2}$	Winter	3.1	1.0	1.7	1.0	1.7	1.1	1.8	**2.4**	**3.6**	1.8	3.0
III		Spring	**3.6**	1.2	**2.5**	**1.8**	**3.6**	**1.5**	**3.0**	2.0	3.4	**2.5**	**4.9**
III		Summer	1.8	1.0	1.0	1.0	1.0	1.0	1.0	2.3	2.0	1.0	1.0
III		Autumn	2.1	**1.4**	1.6	1.4	1.6	1.3	1.5	1.0	1.0	1.0	1.2

In the left column for each parameter, the value 1 is given in each size-range to the smallest response to an equal mass dose (10 mg/kg) of coarse ($PM_{10-2.5}$), fine ($PM_{2.5-0.2}$ or $PM_{2.5-1}$), accumulation ($PM_{1-0.2}$) and ultrafine ($PM_{0.2}$) particulate samples given intratracheally to mice. In the right column, a similar comparison of inflammatory activity is made on the basis of this bioassay for particulate mass per cubic meter of urban air in the sampling campaigns.The largest value for each parameter is shown in bold.

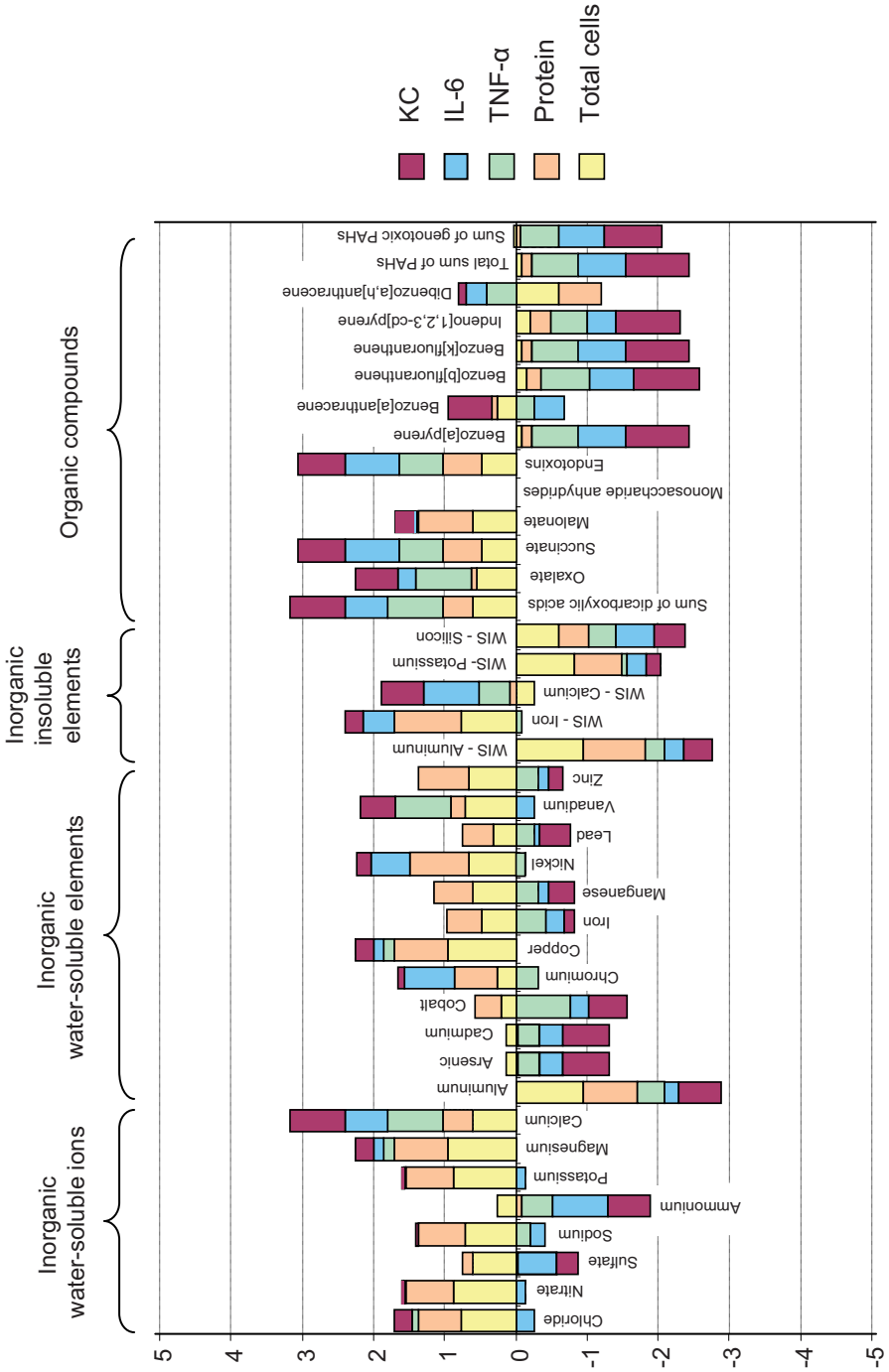
There were fewer statistically significant or nearly significant correlations between chemical constituents and inflammatory responses in $PM_{10-2.5}$ size-range compared to $PM_{2.5-0.2}$ size-range (II). Inorganic ions had both positive and negative associations with the inflammatory responses. Water-soluble elements had frequently positive associations with most of the response parameters. Similarly to $PM_{2.5-0.2}$ size-range, dicarboxylic acids correlated positively and PAH-compounds negatively with the inflammatory markers to the $PM_{10-2.5}$ samples. Endotoxin displayed positive correlations with inflammatory markers in $PM_{10-2.5}$, where the endotoxin content was also higher than in samples in the smaller particle size-ranges.

In the $PM_{2.5-0.2}$ size-range (II), there were both strong positive and strong negative correlations between the chemical constituents and the inflammatory responses. Some ions, i.e. NO_3^-, NH_4^+ and K^+, had negative correlations with the inflammatory markers, whereas Ca^{2+} exhibited positive correlations. SO_2^{4-} displayed no correlation with any of response parameter. Several transition metals, most evidently Ni and V, had positive correlations with the inflammatory responses to the $PM_{2.5-0.2}$ samples. All the dicarboxylic acids had high positive correlations with the inflammatory parameters. In contrast, ΣMA and most of the detected PAH compounds exhibited strong negative correlations with the inflammatory responses.

In the seasonal campaigns in Helsinki (III), much fewer chemical constituents and sources were associated with the inflammatory responses, when compared to the campaigns in the six European cities. Most evidently, resuspended soil material and other non-exhaust particulate material from traffic were associated with the inflammatory responses to the $PM_{10-2.5}$ and the $PM_{2.5-1}$ samples. In the smaller particulate size-ranges, $PM_{1-0.2}$ and $PM_{0.2}$, no strong or consistent associations were found due to the failure of these particles to evoke inflammatory responses.

In repeated dosing of the $PM_{10-2.5}$ and $PM_{2.5-0.2}$ samples from six European cities (IV), the chemical constituents and sources associated with the inflammatory responses in BALF were to a large extent rather similar to those observed in the single dose study (II).

Figure 9. ***Spearman correlation coefficients (ρ) between the selected chemical constituents in $PM_{10-2.5}$ samples from six European cities and the inflammatory response markers in BALF at their most feasible time-points for recording (4 h for cytokines and 12 h for total cells and protein)***



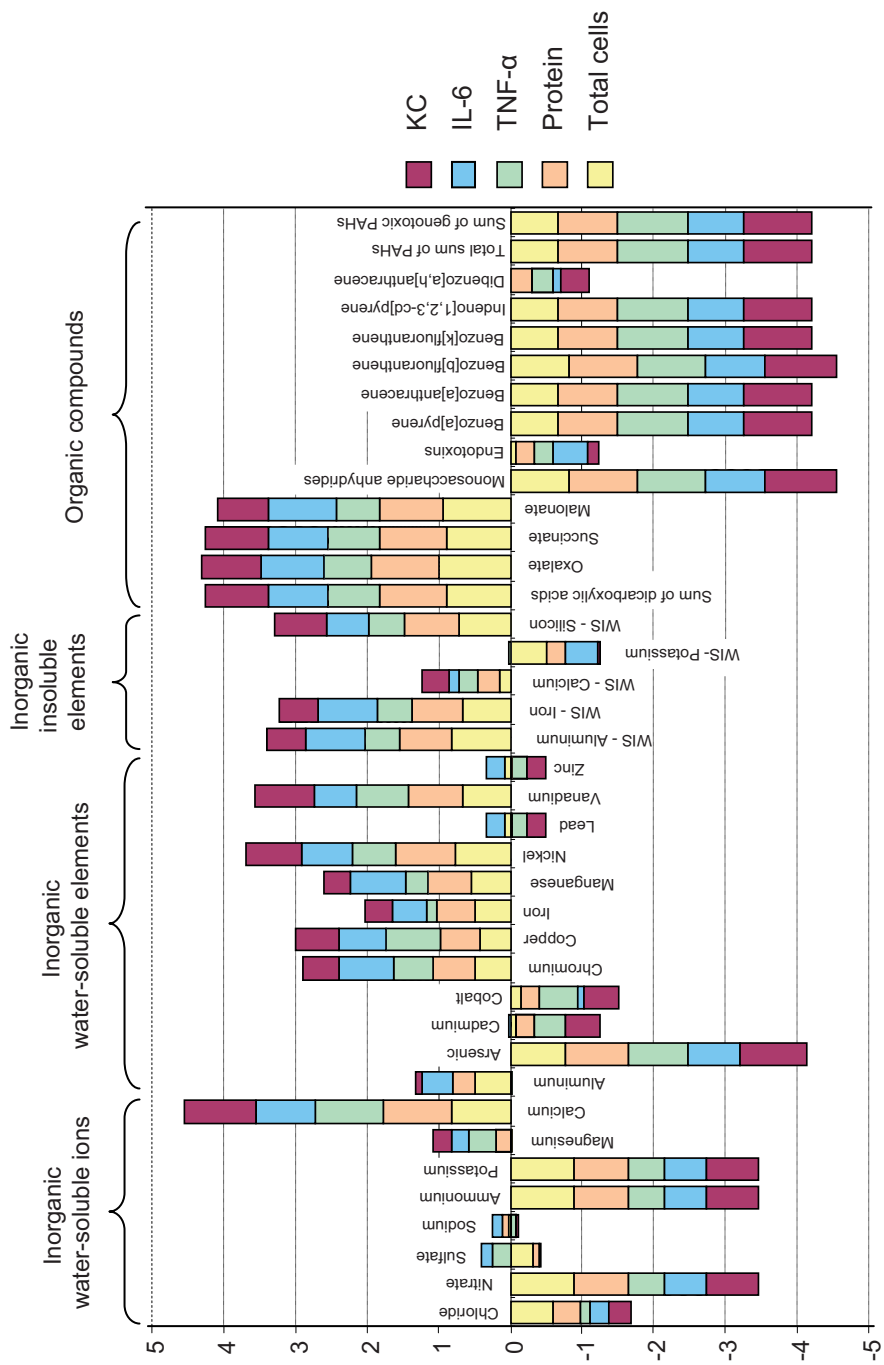


Figure 10. ***Spearman correlation coefficients (ρ) between the selected chemical constituents in PM 2.5-0.2 samples from six European cities and the inflammatory response markers in BALF at their most feasible time-points for recording (4 h for cytokines and 12 h for total cells and protein)***

6 DISCUSSION

There were clear heterogeneities in the inflammatory activities of the urban air particulate samples in different size-ranges with regard to both the magnitude and time course of the responses. Coarse particulate samples displayed generally the highest inflammatory activities of all the samples in the mouse lung, but fine particulate samples showed the largest heterogeneity in this activity between sampling campaigns. Ultrafine particles induced only negligible responses. Moreover, there were significant associations between the inorganic water-soluble and water-insoluble constituents, as well as the organic constituents of coarse and fine particulate samples with the measured inflammatory markers. The local sources of incomplete combustion and resuspended road dust were identified as major contributors to the inflammatory activity of fine particulate samples in mouse lung. Moreover, both fine and coarse particulate material in urban air was assessed to display their highest inflammatory potential in warm and dry environments where there was intense sunlight.

6.1 Pulmonary responses to particulate samples

6.1.1 Dose- and time-dependency (I, II, III, IV)

There were dose-dependent increases of inflammatory markers in BALF at 24 hours after intratracheal instillation of the $PM_{10-2.5}$ samples. In contrast, only the highest dose (10 mg/kg) of the $PM_{2.5-0.2}$ samples caused significant responses. Meanwhile, no consistent response pattern was found to any of the $PM_{0.2}$ samples. No signs of lung overloading with the largest mass dose were observed. Moreover, the doses used in this study were no higher than those usually used to induce inflammatory responses in rodent lungs (Adamson et al. 1999; Walters et al. 2001; Schins et al. 2004, Gerlofs-Nijland et al. 2005 and 2007). Therefore, the largest dose 10 mg/kg in Study I was selected to be used in the rest of the studies for the more detailed investigation of the factors contributing to the inflammatory activity of the particulate samples.

One of the first steps in acute pulmonary inflammation is the production of inflammatory mediators, such as cytokines and chemokines, by the alveolar macrophages, respiratory epithelial cells and neutrophils. In Study I, the cytokine concentrations in BALF followed a clearly more rapid response pattern than the other measured parameters. Large increases in cytokine and chemokine

concentrations in BALF were consistently measured already at 4 hours after exposure to particulate samples, this being followed by a rapid decline.

In several recent studies, cytokine concentrations have been considered as insensitive markers of particulate induced inflammation in rodents (Dick et al. 2003a; Schins et al. 2003; Gavett et al. 2003). The reported exiquity in the cytokine response may be largely due to too late time-points having been selected for cytokine measurements. According to present findings (I), the 4-hour time point seems to be optimal for BALF collection for TNF-α, IL-6 and KC measurements. However, there are studies, which have reported increases in BALF cytokine concentrations at 18-24 hours after exposure to particulate samples, especially those in the coarse size-range (Schins et al. 2004; Gilmour et al. 2007; Hutchison et al. 2005). This prolongation of cytokine response may be at least partially due to a higher proportion of insoluble material in $PM_{10-2.5}$ samples compared to $PM_{2.5-0.2}$ samples as observed in the chemical mass closure assessment for the six European cities samples (II). This may have facilitated particle uptake by inflammatory cells that already exists in the lungs such as macrophages (Kendall et al. 2002). Moreover, it has been shown that cytokine responses are dominated by the insoluble fraction of urban air $PM_{10-2.5}$ and $PM_{2.5-0.2}$ samples in a mouse macrophage cell line (Jalava et al. 2008).

Shortly after excretion of inflammatory mediators, new inflammatory cells migrate into the lungs. In the present studies, this was generally seen with $PM_{10-2.5}$ and $PM_{2.5-0.2}$ samples (I) as well as with $PM_{10-2.5}$ and $PM_{2.5-1}$ samples (III) at 12 hours after a single dose. In the acute phase of inflammation, this increase in the cell number was mostly caused by migration of neutrophils into the lungs (I, IV), which has also been seen in previous studies (Adamson & Prieditis 2004; Gavett et al. 2003, Gerlofs-Nijland et al. 2005; Schins et al. 2003; Wegesser & Last 2008). This supports the view that the number of neutrophils in BALF is a more sensitive indicator of proinflammatory effects than simply counting the total cell number (Wegesser & Last 2008).

Neither total protein nor LDH concentration in BALF showed any systematic time-dependency, which points to a low acute cytotoxic potency of the urban air particles in the mouse lung (I). Similar findings have been reported also by other workers (Dick et al. 2003a; Gerlofs-Nijland et al. 2005).

According to the present studies, an awareness of the optimal time-points of response recording for the key parameters is critical if one wishes to make a reliable assessment of the inflammatory activity of particulate samples in different size-ranges. For cytokine and chemokine measurements in mouse BALF, the optimal time point was 4 hours after exposure to particulate samples, while it was 12 hours

for the assessment of neutrophilic inflammation. In the literature, the most commonly used time points for the analyses of inflammatory markers in BALF have been 18-24 hours after exposure. The present results indicate that the 24-hour time-point was not optimal for analysis of any of the parameters in single-dose studies.

In the present studies (I, III), the inflammatory responses measured at their optimal time-points of detection displayed strong intercorrelations, whereas no such correlations were found between responses at other time-points. The strong correlation between the MIP-2 concentration and the neutrophil number in BALF at the early phase of inflammation, which gradually decreased at the later time points, has also been reported recently in the study of Wegesser & Last (2008). Thus, an optimal time for each detected marker should be used instead of one compromised time-point for all to enable a reliable comparison of the inflammatory activity of urban air particulate samples.

6.1.2 Effect of repeated dosing (IV)

The sub-acute phase of inflammation was studied by repeated dosing of particulate samples from the European six-city campaigns. The results showed only slight increases of proinflammatory cytokines from the control level after repeated dosing. However, total cell numbers in BALF were clearly increased, mostly because of a larger proportion of macrophages. Similarly to the effects of single dose, $PM_{10-2.5}$ samples showed higher inflammatory activity than $PM_{2.5-0.2}$ samples after repeated dosing. No signs of particle overloading or free particles in the extracellular space of the lungs were observed after repeated dosing with any of the samples.

In contrast to single dose studies, there were some mild but consistent signs of cytotoxicity detected after repeated dosing of the particulate samples. The LDH concentration in BALF was elevated after repeated dosing of $PM_{10-2.5}$ samples, which is interpreted as evidence of cell damage in the mouse lungs. In addition, more extensive inflammatory lesions by $PM_{10-2.5}$ samples compared to $PM_{2.5-0.2}$ samples were confirmed in the histopathological examination of the lungs. Overall, $PM_{2.5-0.2}$ samples seemed less cytotoxic than $PM_{10-2.5}$ samples, which is at odds with the results of Jalava et al. (2007) in the macrophage cell line.

Steerenberg et al. (2004) have also previously shown that $PM_{10-2.5}$ samples induce more extensive inflammatory lesions in the mouse lungs than $PM_{2.5-0.2}$ samples. Gerlofs-Nijland et al. (2005 and 2007) have reported that the severity of lesions, such as alveolitis, increased cell proliferation, bronchiolitis and other inflammatory foci, in the rat lungs is dependent on the mass dose of intratracheally instilled particulate samples.

6.2 Particulate sample induced responses

There were considerable heterogeneities in the inflammatory activities of particulate samples in the different size-ranges and sampling campaigns. However, it should be noted that samples collected from the six European cities do not specifically represent any city or season due to the short sampling periods. They rather represent size-segregated particulate matter in selected source environments that were to varying degrees affected by common local emission sources and seasonal factors. Seasonal variation of particulate induced inflammatory activity was studied in Helsinki during the four seasons of the year, which are quite distinct in terms of temperature, rainfall and solar radiation.

6.2.1 Particle size (I, III, IV)

The comparisons of inflammatory activities in BALF of particulate samples in different size-ranges were made at the most optimal time points of the response. The $PM_{10-2.5}$ samples were the most potent inducers of inflammatory responses (I, III), which has been noted in several other *in vivo* studies (Schins et al. 2004; Steerenberg et al. 2004; Gerlofs-Nijland et al. 2007; Gilmour et al. 2007; Jalava et al. 2006). Moreover, $PM_{10-2.5}$ samples induced more extensive inflammatory lesions in the lung tissue than the $PM_{2.5-0.2}$ samples (IV).

There were relatively minor differences in the inflammatory activity between the $PM_{10-2.5}$ samples from six European campaigns, whereas a relatively great heterogeneity was observed in the inflammatory activity between the $PM_{2.5-0.2}$ samples. However, both the largest total cell number and the cytokine concentrations in BALF were equally high in both of the size-ranges, if detected at their most optimal time-points (I).

Urban air $PM_{0.2}$ samples had negligible inflammatory activities (I, III), which is in agreement with the findings of other in vivo and in vitro studies (Kooter et al. 2006; Becker et al. 2003 and 2005; Jalava et al. 2006).

6.2.2 Source environment (I, II)

The toxic activities of particulate samples within the $PM_{10-2.5}$ and $PM_{2.5-0.2}$ size-ranges were compared on an equal mass basis and per cubic meter of ambient air. These calculations were performed to create a connection to the real-life particulate pollution situation impacting on the individual's respiratory tract.

The warm and dry season sampling campaigns of Barcelona and Athens had the highest overall inflammatory activities in both the $PM_{10-2.5}$ and $PM_{2.5-0.2}$ size-ranges. In $PM_{10-2.5}$, this was obviously due to the highest particulate mass concentrations of all campaigns and the relatively high inflammatory activity of the particles per unit of mass. $PM_{2.5-0.2}$ samples exhibited the highest atmospheric photochemical transformation of organic compounds, as indicated by the dicarboxylic acid contents. This is postulated to be due to the great contribution of oil combustion derived organic compounds and transition metals (Ni, V), originating from the local large harbours (Sillanpää et al. 2005). Moreover, non-combustion emissions from vehicles, such as brake and tyre dust as well as mineral-rich road dust, have most likely contributed to the high inflammatory activity of both the $PM_{10-2.5}$ and the $PM_{2.5-0.2}$ samples from Barcelona and Athens. However, endotoxin levels of the Barcelona and Athens samples were not significantly higher than those measured from the samples of other cities. In previous studies, traffic has been associated with adverse biological and histopathological effects in the rat lung and vascular system (Gerlofs-Nijland et al. 2007). Seagrave et al. (2006) have also claimed that traffic and industry are the two most toxic sources of ambient air particles.

In contrast to Mediterranean sampling campaigns, the samples gathered in the cold and wet season samplings in Duisburg, Prague and Amsterdam displayed most often much lower inflammatory activities, especially with regard to $PM_{2.5-0.2}$ samples. Particularly in the Prague campaign, one explanation is the heavy use of coal and biomass combustion for domestic heating, which are two sources likely to account for the high levels of PAH-compounds measured in these samples (Saarnio et al. 2008). PAH-compounds have evoked immunosuppressive effects, e.g. a decrease in cytokine production in the rat lungs (Kong et al. 1994). It has also been suspected that PAH compounds in fine particulate samples cause cell cycle arrest in the G2/M phase of mouse macrophages (Jalava et al. 2007) and in this way can evoke immunosuppressive effects. In the present study, the lowest PAH contents of the $PM_{2.5-0.2}$ samples were measured in Mediterranean campaigns, which may be due to a rapid photo-oxidation of these compounds in the prevailing atmospheric conditions (WHO IPCS 1998).

6.2.3 Seasonal variation (III)

The seasonal heterogeneity in the toxic activities of size-segregated urban air particulate samples was studied by collecting samples during four seasons from the same site in Helsinki. The relative inflammatory activities per mass unit of the $PM_{10-2.5}$ and $PM_{2.5-1}$ samples were at their highest in the autumn campaign. One possible explanation for this phenomenom is that the microbe concentrations present in the

outdoor air are known to be relatively high in the autumn and summer in Finland (Kaarakainen et al. 2008). However, when adjusted to the urban air mass concentration, the highest inflammatory potential in the seasonal campaigns was obtained for particulate matter collected during the spring. This outcome was greatly influenced by the larger springtime particulate mass concentrations in these two size ranges attributable to resuspended road dust. However, the highest relative inflammatory activities per mass unit of the $PM_{1\text{-}0.2}$ and $PM_{0.2}$ samples were most often observed in the spring, but in these size ranges there was more variability in the different parameters between the seasons than with the samples in larger size ranges. The same applied to the overall inflammatory potential per cubic meter of urban air in the seasonal campaigns. It is possible that in a similar manner to the finding in the Mediterranean $PM_{2.5\text{-}0.2}$ samples, solar radiation and the resultant photo-oxidation of organic compounds increased the inflammatory activity of these particulate samples. In previous cell studies (Dybing et al. 2004; Becker et al. 2005, Jalava et al. 2006), particulate samples collected during warm seasons have had higher inflammatory activities than samples collected in colder seasons.

6.3 Sources and chemical compositions responsible for pulmonary effects

6.3.1 Coarse particles (II, III)

Coarse particles are mostly derived from local sources. The most abundant of the identified components of $PM_{10\text{-}2.5}$ samples were soil-derived minerals in the spring and summer campaigns (II). In Helsinki, the major local source for crustal material was resuspended road dust, whereas in the Mediterranean cities, also dust storms from the Sahara may have contributed to the particulate mass (Sandström and Forsberg 2008). Moreover, there can be high contributions by biogenic material and sodium chloride (sea and road salt) in the samples of autumn and winter campaigns (Jones and Harrrison 2004; Sillanpää et al. 2006).

There was less heterogeneity within the $PM_{10\text{-}2.5}$ samples compared to the $PM_{2.5\text{-}0.2}$ samples (I) in terms of both their chemical composition and the inflammatory responses they evoked. Moreover, there were also fewer significant correlations found between the chemical constituents and the measured responses. Soil derived constituents, such as Ca^{2+}, Fe, Mn and insoluble Fe, were associated with increased inflammatory responses. However, there were also some negative associations of Al, K, and Si with the inflammatory responses. This may indicate that some reactive constituents, attached to or otherwise co-appearing with certain type of mineral

particles, are the true causative or dominating agents responsible for the responses. One candidate culprit is the endotoxins from gram-negative bacteria, which have a role in the inflammatory effects of coarse particles (Becker et al. 2003; Salonen et al. 2004; Alexis et al. 2006). However, the causative role of endotoxins remains obscure in the present studies. The endotoxin concentration in the European six-city samples showed only some nearly significant positive correlations with the inflammatory markers, and no association was found with the seasonal samples of Helsinki. A second candidate is soluble Fe attached to the surfaces of mineral particles (Ghio et al. 1992; Hetland et al. 2000). The results from the European six-city particulate samples did not clearly support this hypothesis, but in contrast, the inflammatory responses to the seasonal samples from Helsinki did reveal some positive associations with soluble Fe. A third candidate to account for the differences in inflammatory activity is the local mineral compositions (Becher et al. 2001). Moreover, different shapes of mineral particles may also be an influencing factor (Holopainen et al. 2004).

6.3.2 Fine particles (II, III)

Fine particles are a mixture of local sources and long-range transported aged particles. The largest differences in the chemical composition and in the induced inflammatory responses were found within the $PM_{2.5-0.2}$ size range (I). Particulate organic matter was generally the largest component in fine particles, followed by secondary inorganic ions. Furthermore, there was also a small contribution from soil derived mineral components.

PAH compounds from biomass and coal combustion displayed consistently negative correlations with the inflammatory markers in BALF, and were, therefore, regarded as having immunomodulating effects on the respiratory defence system (II). This view is also supported by an *in vitro* study where the same fine and ultrafine particulate samples were tested in a mouse macrophage cell line (Jalava et al. 2007). Moreover, wood smoke-rich particulate samples have apparently a low inflammatory activity also in *in vivo* studies, which could be due to an immunosuppressive effect of PAHs (Seagrave et al. 2006). However, there was no clear negative correlation between the PAH compounds and the inflammatory responses in relation to the seasonal samples of Helsinki. One reason why it was not possible to observe the anticipated correlation pattern may be the much smaller, 4-fold range of genotoxic PAH contents between the seasonal $PM_{1-0.2}$ samples of Helsinki compared to the 23-fold range between the $PM_{2.5-0.2}$ samples collected in the six European cities (Saarnio et al. 2008).

In contrast to PAHs, dicarboxylic acids exhibited positive associations with all the measured inflammatory markers. Interestingly, the contents of dicarboxylic acids and PAHs had a high inverse correlation with each other, which suggests that they have opposing generation or breakdown pathways in different seasons. The dicarboxylic acids represent hydrophilic, low-reactivity end products created from a complex photochemical transformation process of combustion-related organic compounds. Thus, their content in fine particulate samples is elevated during sunny seasons (Kawamura and Ikushima, 1993). In contrast, PAH compounds are known to have much shorter half-times in the atmosphere during warm and sunny seasons compared to cold and dark seasons, which is due to their photo-oxidation (WHO-IPCS 1998). The highest levels of dicarboxylic acids in six European cities were measured during the warm and sunny campaigns (Sillanpää et al. 2005), when the PAH concentrations were at their lowest (Saarnio et al. 2008). Thus, it is possible that the higher inflammatory activity of the Barcelona and Athens samples was, at least partially, due to the breakdown products of PAHs, i.e. quinoid substances that are hypothesized to be efficient producers of reactive oxygen species (Squadrito et al. 2001).

Source tracers for the combustion of fuel oil, Ni and V, had consistent positive associations with several inflammatory markers. Their concentrations were at their highest in Barcelona and Athens, which suggests that ships in large local or regional harbors had contributed to the fine particulate composition (Sillanpää et al. 2005). At the same time, V and Ni may causally contribute to the induction of inflammation via redox in a reactions similar manner to the other transition metals (Dye et al. 2001; Gavett et al. 1997; Hutchison et al. 2005; Rice et al. 2001). In addition, several other transition metals also showed positive correlations with some of the inflammatory markers. Their sources are mainly traffic and large metal industries.

In addition, soil derived constituents were positively associated with the inflammatory responses to $PM_{2.5-0.2}$ samples. The soil-derived material not only appears in coarse size range, but also to some extent in fine particles (Sillanpää et al. 2006).

6.3.3 Accumulation mode and ultrafine particles (II, III)

Ultrafine particles in urban air are mostly derived from local combustion sources. The accumulation mode largely consists of ultrafine particles that have coagulated with each other. Urban air $PM_{1-0.2}$ and $PM_{0.2}$ samples evoked only very small inflammatory responses. Therefore, their associations with particulate constituents and sources should be considered more cautiously than those in connection to PM_{10-}

$_{2.5,}$ $PM_{2.5-1}$ and $PM_{2.5-0.2}$ samples. The greatest components of the $PM_{1-0.2}$ and $PM_{0.2}$ sample mass, i.e. SO_4^{2-}, NO_3^- and NH_4^+, displayed no consistent correlation patterns with the responses. This agrees well with the general concept of their low toxic potency (Schlesinger and Cassee 2003).

The low inflammatory activity of $PM_{0.2}$ samples may not be simply due to the methodological reasons, i.e. removal of fibreglass filter fragments and simultaneous loss of solid carbon particles from the methanol extract. This is supported by the fact that also $PM_{1-0.2}$ samples had a very low overall inflammatory potential. These samples were collected on PUF strips and the particulate material was extracted for toxicological experiments in the same way as the more potent $PM_{10-2.5}$ and $PM_{2.5-1}$ samples. Thus, the particle-size dependent positive relationship with the inflammatory activity of urban air samples may well be due to the higher proportion of insoluble material in the larger size ranges.

6.4 Methodological considerations

This thesis indicates that important new information can be obtained on the contributions of different sources and constituents to urban air particle-related inflammatory activity by combining single-dose animal study results with an in-depth chemical characterization of the particulate samples. The availability of extensive chemical data from particulate samples collected with two different sampler types certainly has its benefits, e.g. a larger proportion of the particulate mass could be identified. There are also weaknesses i.e. different sampler characteristics. However, according to recent methodological evaluation (Pennanen et al. 2007) on the same particulate samples as used in the present studies, the mass of both the HVCI $PM_{10-2.5}$ and $PM_{2.5-0.2}$ particulate samples could be collected and extracted at high efficiency. In addition, the contents of chemical constituents (with the exception of SO_4^{2-} and NH_4^+) in the HVCI samples correlated reasonably well with those in parallel low volume reference samples.

A controlled intratracheal instillation technique was used in the present studies to achieve the delivery of the size-segregated particulate samples to the lower airways of mice. Naturally, this exposure differs from the inhalation exposure to actual concentrated ambient air particles (CAP). However, the two exposure techniques have resulted in semiquantitatively similar particulate distribution patterns and inflammatory responses in rodent lungs (Driscoll et al. 2000; Costa et al. 2006). Testing several dose-levels and time-points would not be possible with CAPs obtained directly from ambient outdoor air, because the aerosol properties are continuously changing. Furthermore, a re-aerosolization of previously collected particles would not have been feasible in the present studies, as it would require

much larger amounts of particulate mass than that available for the instillation technique.

The doses of particulate matter used in the present studies may seem relatively high, when compared to doses that are likely to be delivered acutely to the lungs in CAP studies. However, these doses are no higher than the instilled doses that have usually induced inflammatory responses in rodent lungs according to the cited literature (Adamson et al. 1999; Walters et al. 2001; Schins et al. 2004; Gerlofs-Nijland et al. 2005). The use of these kinds of doses is necessary if one wishes to demonstrate statistically significant differences in the inflammatory activity between the particulate samples. This is especially true for small groups of healthy animals used in the present studies. Moreover, not all particulate matter instilled to the trachea reaches the deep lung but part of it is removed via mucociliary clearance as is the case with inhalation. Animals with pre-existing chronic lung disease may be generally more sensitive to the inflammatory effects of particulate samples, but the disease status would contribute additional variability to responses. In any case, there were no signs of lung overloading with the largest mass dose of 10 mg/kg even in the repeated dose study. It has been suggested however, that the use of somewhat lower doses may be possible when the best and optimal time points for each measured parameter are used in response recording.

The results of the present studies show clearly the importance of knowing the time course of an acute inflammatory response in different parameters used in the investigation of the inflammatory activities of urban air particulate samples in the mouse lung. It is clear that more than one time point is needed for a reliable assessment of the inflammatory responses. With three time points, it was possible to demonstrate relatively intense inflammatory activities with both coarse and fine particulate samples as well as particle size, dose regimen and sampling campaign-dependent heterogeneities in response profiles.

The number of mice used in the present studies was kept to a minimum for ethical reasons. Therefore only three animals instead of six were exposed to $PM_{0.2}$ samples that produced only small or inconsistent responses. Moreover, only a minimal number of animals were exposed to the positive control sample (EHC-93), because these exposures were made only for quality assurance. Mild general anesthesia was used during intratracheal exposure of the animals to eliminate possible pain and discomfort. Overall, the animals appeared to be in good condition during experiments. There were no changes in their weights or behaviour, even during the repeated-dose study.

7 CONCLUSIONS

1. Heterogeneities in the inflammatory activities of urban air coarse, fine, and ultrafine particulate samples were detected with regard to both the magnitude and duration of responses. Coarse particles displayed the highest inflammatory potency, followed by fine particles. Ultrafine particulate samples induced only negligible responses. Large responses in BALF cytokine concentrations appeared within only a few hours and lasted for a much shorter time than those in BALF inflammatory cell number and total protein concentration. Knowledge of the time-course of responses in the selected parameters is crucial if one wishes to achieve a reliable comparison of the inflammatory activity of urban air particulate samples collected in different locations or seasons. It is clear that more than one time-point is needed for a reliable assessment of the cytokine-dependent acute inflammatory response to urban air particles in the rodent lungs.
2. Oxidized organic compounds and transition metals, especially those from fuel oil combustion, contributed to the inflammatory activity of fine particles. In addition, soil-derived particulate constituents showed similar contributions to the inflammatory activity of fine particulate samples, but their role in coarse particulate samples was not so obvious, possibly due to a contribution of largely undefined biogenic material. The regionally and long-range transported secondary inorganic ions had either negative or inconsistent associations with the inflammatory activity. Poor combustion of biomass and coal was associated with elevated PAHs contents and a consistent immunosuppressive effect of fine particulate samples.
3. Coarse particulate samples collected during four seasons in Helsinki had higher inflammatory activities than the corresponding urban air particulate samples in smaller size-ranges. Fine particulate samples collected during warm and dry seasons had higher inflammatory activity than the samples collected during winter. Resuspension of road dust in springtime caused a substantial increase in the seasonal urban air particle mass concentration, especially in the coarse size-range. Moreover, resuspended road dust and other non-exhaust particulate material from traffic contributed to the increased inflammatory activity of the coarse and intermediate size-range samples collected in the spring and autumn. Photo-oxidation of combustion derived organic compounds had some positive associations with the inflammatory activities of particulate samples in accumulation and ultrafine size-ranges.

4. Repeated intratracheal dosing of particulate samples in both coarse and fine size-ranges caused an accumulation of inflammatory cells and cytotoxicity in the mouse lungs compared to single-dose exposure to the same samples. In addition, the inflammatory lesions in the lung tissue after repeated dosing were more extensive than those encountered after a single dose. Coarse particulate samples displayed stronger inflammatory and cytotoxic activities than fine particulate samples, similar to the situation after a single dose. However, cytokine concentrations in BALF after repeated dosing were systematically lower than those seen after single dose. Mediterranean sampling campaigns, and the same constituents and sources as in the single dose study, were associated with inflammatory activities of the fine and coarse particulate samples in the mouse lung after repeated dosing.

Thus, local sources of oil combustion, and traffic-derived brake and tyre dust, and resuspended mineral dust are likely to contribute to the inflammatory activity of urban air fine and coarse particles in conjunction with meteorological factors such as the intensity of sunlight, temperature and rain. Products from incomplete wood and coal combustion, especially PAHs, evoke immunosuppression.

The present results have contributed to a deeper understanding of the sources and constituents in the complex mixture of particulate air pollution which are potentially hazardous to people living in urban environments. These results can help explaining the results of epidemiological studies and be utilized in the future evaluations of the health risks associated with particulate air pollution.

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